

ENVIRONMENTAL DETECTION OF BACULOVIRUS SPECIES AND
GENOTYPES USING THE POLYMERASE CHAIN REACTION TECHNIQUE

By

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To my dear parents and loving husband.

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TABLE OF CONTENTS

	<u>page</u>
ACKNOWLEDGMENTS	iii
LIST OF TABLES	ix
LIST OF FIGURES	x
ABSTRACT.....	xii
1 INTRODUCTION	1
Literature Review.....	1
Baculoviruses-General	1
Classification and morphology	1
Life cycle	3
Genomic organization.....	5
Structural proteins: Polyhedra-derived phenotype.....	6
Regulatory proteins.....	10
Proteins involved in viral DNA replication	10
Homologous repeats.....	11
Importance of Baculoviruses	12
Use of baculoviruses as microbial control agents.....	12
Use of baculoviruses as expression vectors	15
Genetically-engineered Baculoviruses.....	16
Ecology of Baculoviruses	19
Persistence.....	20
Dispersal	22
Transmission	24
Detection of Baculoviruses	25
Present Study	29
2 DETECTION AND IDENTIFICATION OF MULTIPLE BACULOVIRUSES.....	31
Introduction.....	31
Materials and Methods.....	34
Viral DNA.....	34

Primer Design	34
PCR Conditions	35
Restriction Enzyme Analysis	36
Results	36
Discussion	41
 3 USE OF POLYMERASE CHAIN REACTION TO MONITOR AND TO STUDY PERSISTENCE OF THE <i>ANTICARSIA GEMMATALIS</i> MULTIPLE- NUCLEOPOLYHEDROVIRUS IN SOYBEAN FIELDS	46
Introduction.....	46
Material and Methods	49
Virus.....	49
Field Conditions.....	50
Viral DNA Extraction from Individual Larvae and Individual Predators.....	52
PCR Conditions	52
Primers	53
Sensitivity of polyhedrin PCR	54
Statistical Analysis.....	54
Results.....	55
Sensitivity of Polyhedrin PCR.....	56
Detection and Environmental Fate of AgMNPV	56
Detection of AgMNPV genotypic variants in Soybean Fields	62
Discussion	68
 4 <i>ANTICARSIA GEMMATALIS</i> BACULOVIRUS DETECTION FROM SOIL SAMPLES	74
Introduction.....	74
Material and Methods	77
Polyhedral Extraction and Viral DNA Purification	77
Phenol-ether extraction	77
Magnetic capture-hybridization (MCH)	78
PCR Conditions	79
Collection of Field Soil Samples	80
Competitive PCR (cPCR).....	80
Construction of Competitor DNA.....	81
Pretreatment of PCR product.....	81
Restriction enzyme digest.....	82
Ligation of fragments.....	82
Cloning.....	83
Transformation of <i>E. coli</i> DH5 α competent cells.....	83
Glycerol stock from positive clones	84
Plasmid DNA purification and confirmation of positive clones.....	84
Test for Heteroduplex Formation.....	85

Test for Differences in Amplification Efficiencies	85
Standard Curve Construction	86
Competitive PCR Product Visualization and Analysis	87
NuSieve gel electrophoresis and alkaline blotting	87
Prehybridization, hybridization, and washes	87
PhosphorImager analysis	88
Efficiency of DNA Isolation Procedure	88
Results	89
Sensitivity of DNA Extraction Procedures	89
Detection of AgMNPV DNA from Field Soil Samples	92
Competitive PCR	92
Efficiency of the MCH Procedure	100
Discussion	112
SUMMARY AND DIRECTION OF FUTURE RESEARCH	120
APPENDICES	
A PURIFICATION OF POLYHEDRA FROM INFECTED LARVAE	125
B PURIFICATION OF THE ALKALI RELEASED VIRUS	126
C BACULOVIRUS DNA PURIFICATION	127
D POLYHEDRIN PCR PROTOCOL	128
E VIRAL DNA EXTRACTION PROTOCOL FOR INDIVIDUAL LARVAE AND INDIVIDUAL PREDATORS	129
F POLYHEDRA EXTRACTION FROM SOIL AND VIRAL DNA PURIFICATION BY PHENOL-ETHER EXTRACTIONS	130
G MAGNETIC CAPTURE-HYBRIDIZATION (MCH)	132
H POLYHEDRA EXTRACTION FROM SOIL AND VIRAL DNA PURIFICATION TO BE USED IN THE MCH PROCEDURE	133
I POLYHEDRIN PCR PROTOCOL FOR AGMNPV DNA FROM SOIL SAMPLES	134
J COMPETITIVE POLYHEDRIN PCR PROTOCOL	135
K ALKALINE BLOTTING	136
L DNA LABELING BY THE NICK TRANSLATION WITH MINIMAL DNASE METHOD	138

LIST OF REFERENCES	140
BIOGRAPHICAL SKETCH	164

LIST OF TABLES

<u>Table</u>	<u>page</u>
2.1: Oligonucleotide primers sequence and sequence similarity of the respective viral DNAs.	38
2.2: Expected fragments obtained after restriction digestion of PCR amplified viral DNAs	40
3.1: Specificity and sensitivity of AgMNPV detection at the intraspecific level	68
4.1: Validation of the standard curve using 1.0 pg of competitor DNA.	99
4.2: Ratios between AgMNPV polyhedrin target and 510 bp competitor PCR products and “calculated” target concentrations using 100 pg of competitor DNA.....	106
4.3: Ratios between AgMNPV polyhedrin target and 510 bp competitor PCR products and “calculated” target concentrations using 50 pg of competitor DNA.....	107
4.4: Validation of the standard curve using 100 pg of competitor DNA.	111
4.5: Validation of the standard curve using 50 pg of competitor DNA.	111

LIST OF FIGURES

<u>Figure</u>	page
2.1: Specific PCR amplification of the <i>polyhedrin</i> gene coding region of four baculoviruses.....	39
2.2: Restriction profile of eight baculovirus PCR products of the <i>polyhedrin</i> gene coding region digested with Hha I.....	42
2.3: Restriction profile of eight baculovirus PCR products of the <i>polyhedrin</i> gene coding region digested with Hinc II.....	43
3.1.: Schematic Representation of Field Design.....	51
3.2: Sensitivity of polyhedrin PCR in the presence and in the absence of larval extracts.....	57
3.3: AgMNPV-2D polyhedrin PCR products uncut (un) and HincII profiles (Hc) for field-collected <i>A. gemmatilis</i> larvae.....	58
3.4: Intensity of polyhedrin PCR products obtained from <i>A. gemmatilis</i> larvae collected at five different sampling dates from AgMNPV-treated soybean plots.....	60
3.5: Percent PCR detection of the <i>Anticarsia gemmatilis</i> nuclear polyhedrosis virus (%) in its larval host, <i>A. gemmatilis</i> , monitoring the <i>polyhedrin</i> gene.....	61
3.6: Percent PCR detection of the <i>Anticarsia gemmatilis</i> nuclear polyhedrosis virus (%) in <i>Nabis</i> spp. and <i>Geocoris</i> spp.....	63
3.7: PCR amplification of the highly variable region 4 for the AgMNPV genomic variants 2D and D7 and seven other baculovirus species.....	64
3.8: Percent PCR detection of the <i>Anticarsia gemmatilis</i> nuclear polyhedrosis virus (%) in its larval host, <i>A. gemmatilis</i> , monitoring the hr4 region.....	65
3.9: Detection of AgMNPV genotypes by PCR targeting the hr4 region.....	67
4.1: Detection limit of the phenol-ether procedure to isolate AgMNPV DNA from soil.....	91
4.2: Detection limit of the magnetic capture-hybridization procedure to isolate AgMNPV DNA from soil.....	93

4.3: Percent PCR detection of the <i>Anticarsia gemmatilis</i> nucleopolyhedrovirus in soil samples.....	94
4.4: PCR amplification efficiency test	96
4.5: Test for heteroduplex formation.	97
4.6: (A) Range of amounts of target DNA (AgMNPV DNA) co-amplified with 1 pg of competitor DNA. (B) Standard curve for competitive PCR of the AgMNPV <i>polyhedrin</i> gene.....	98
4.7: Accuracy of AgMNPV DNA quantitation by competitive PCR.	101
4.8: Competitive polyhedrin PCR for field-collected soil samples.	102
4.9: cPCR results for AgMNPV DNA isolated from soil using the MCH procedure.	104
4.10: Standard Curve of competitive PCR of AgMNPV <i>polyhedrin</i> gene using 100 pg of competitor DNA.....	109
4.11: Standard curve of competitive PCR of AgMNPV <i>polyhedrin</i> gene using 50 pg of competitor DNA.....	110

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The detection of baculovirus species and genotypes in the environment is extremely important because of the large utilization of wild-type viruses to control insects and the potential use of genetically-improved baculoviruses. The polymerase chain reaction (PCR) was chosen as a detection method due to its high specificity and sensitivity.

Detection of multiple baculovirus species was achieved by choosing the *polyhedrin* gene as target DNA for PCR amplification, because of its highly conserved nature among baculoviruses. PCR for the *polyhedrin* gene detected eight different species of baculoviruses for the following insects *Autographa californica*, *Anticarsia gemmatilis*, *Spodoptera frugiperda*, *S. exigua*, *Bombyx mori*, *Orgyia pseudotsugata*,

Anagrapha falcifera, and *Heliothis zea*. These viruses were distinguished by restriction enzyme analysis of the polyhedrin PCR product with HhaI and HincII.

AgMNPV applied in soybean fields was detected from environmental samples including larval hosts, insect predators, and soil. PCR amplification of the *polyhedrin* gene DNA detected AgMNPV in the host and predator populations from one to 45 days after virus application. The AgMNPV genetic variants, 2D and D7, were applied separately and as a mixture in the field. Detection of these genotypes was accomplished by PCR amplification of a highly variable region, hr4, in the AgMNPV genome, producing PCR products of 1,726 base pairs and 1,345 base pairs for the 2D and D7 genotypes, respectively. A magnetic capture-hybridization procedure was used to isolate baculovirus DNA from soil. This DNA isolation method coupled with PCR amplification of the *polyhedrin* gene DNA, detected AgMNPV from 15 to 180 days post-application.

In conclusion, PCR amplification provided a specific, fast, and sensitive way to detect and to identify baculoviruses in the environment. The use of conserved and variable DNA regions of the viral genome as targets for PCR amplification enabled detection at the species and genotype level. This research will benefit the study of the environmental fate and ecology of baculoviruses, and it could potentially be used in quality control of programs in which baculoviruses are being applied to control insects as well as in quality control of the commercial production of these viruses.

CHAPTER 1 INTRODUCTION

Literature Review

Baculoviruses-General

Baculoviruses are large, double-stranded, circular DNA viruses, which are highly infective to invertebrates. These viruses are found mostly in insects, but they also occur in Crustacea (Couch, 1974; Summers, 1977). Baculoviruses infect over 600 species of insects (Martignoni & Iwai, 1986), mainly from the order Lepidoptera, but also from Hymenoptera, Diptera, Coleoptera, and Trichoptera.

The first description of baculoviruses was made in a poem by the Italian poet and bishop, Marco Girolamo Vida, who described the disease of the silkworm (*Bombyx mori* Linnaeus) in 1527 (Benz, 1986). Historically, the science of baculoviruses has followed a long path, and currently it has expanded from pest control into the field of genetic engineering, where the virus serves as a vector for the expression of foreign genes producing proteins of medical and pharmaceutical importance (Tanada & Kaya, 1993).

Classification and morphology

For a long time it was usual to associate viruses with the host species in which they were isolated for the first time, thus disregarding the possibility that a single virus may infect different hosts (Evans & Entwistle, 1987). Modern techniques allow the

identification of viruses based on type of nucleic acid, structure of the virus particle, and presence or absence of an inclusion body. According to the Sixth Report of the International Committee on Taxonomy of Viruses (Murphy et al., 1995), baculoviruses are classified in the family Baculoviridae, presenting two genera, Nucleopolyhedrovirus and Granulovirus.

Nucleopolyhedrovirus (NPV). The type species in this genus is the *Autographa californica* nucleopolyhedrovirus. In the nucleopolyhedroviruses, the virion is occluded within a polyhedral protein matrix composed mainly of a single protein (polyhedrin). Each occlusion body (OBs) measures 0.15 to 15 mm in size, with either a single nucleocapsid per envelope (single-nucleocapsid nucleopolyhedrovirus, SNPV) or one to several nucleocapsids per envelope (multinucleocapsid nucleopolyhedrovirus, MNPV). Occlusion bodies are formed within the nucleus of infected cells, and many enveloped nucleocapsids (virions) are embedded in each OB. Nucleocapsids are rod-shaped (30-60 nm x 250- 300 nm) and contain a single molecule of circular supercoiled double-stranded DNA, which ranges in size from 90-160 kbp.

Granuloviruses (GVs). The type species in this genus is the *Plodia interpunctella* granulovirus. Granuloviruses usually contain only one nucleocapsid per envelope and a single (occasionally two) virion per OB. The virion is occluded within an ovicylindrical protein matrix composed primarily of a single protein (granulin). Each occlusion body measures 0.13 to 0.5 mm in size. Nucleocapsids are also rod-shaped (30-60 nm x 250- 300 nm) and contain a single molecule of circular supercoiled dsDNA, which ranges in size from 90 to 180 kbp.

Life cycle

In natural conditions and in application of baculoviruses as microbial insecticides, NPVs are found on plant surfaces and in the soil. They must be ingested by their larval hosts in order to begin the infection process. The occlusion bodies, commonly known as polyhedra, are composed of a 29-kd protein called "polyhedrin" (Summers & Smith, 1978), which confer protection to the infectious virions from adverse environmental conditions such as UV light. Baculoviruses are unique because they have a biphasic mode of replication (Volkman et al., 1976). Polyhedra are ingested when the larval host feeds on contaminated leaves. These polyhedra are dissolved in the alkaline midgut environment of the susceptible host, releasing the envelope nucleocapsids (ENCs). ENCs must pass through the peritrophic membrane, which is believed to be a barrier to microbial infection, and reach the microvillar region of the midgut epithelial cells. The viral envelope fuses with the plasma membrane of the epithelial cells and releases the nucleocapsids inside the cytoplasm (Granados, 1978). The nucleocapsids move towards the cell nucleus and uncoating occurs either at the nuclear pore membrane (Paschke & Summers, 1975; Tanada & Hess, 1976) or in the nucleoplasm (Granados, 1978; Granados & Lawler, 1981). The uncoating process is not completely understood at this time. After uncoating, the nucleus becomes enlarged and an electron-dense network called virogenic stroma is formed (Granados & Lawler, 1981; Granados et al., 1981). Assembly of virus particles occurs in the virogenic stroma. Nucleocapsids are formed about eight hours post-infection (h.p.i.) (Granados & Lawler, 1981). During primary replication, which occurs in the infected gut cells, the nucleocapsids bud through the

nuclear membrane (at approximately 12 h p.i.) and acquire a loose-fitting envelope. This envelope is lost in the cytoplasm, and the nucleocapsids bud through the plasma membrane, acquiring an envelope with peplomers at one end (Granados, 1980; Granados & Lawler, 1981; Granados et al., 1981). The peplomers are formed by a glycoprotein of 64 Kda (gp64) which has been inserted into the plasma membrane (Volkman & Goldsmith, 1984). This glycoprotein probably serves to attach the budded virus (BV) to other susceptible cells. The budded virus (BV), also called extracellular virus (ECV), is released into the hemolymph and infects other cell types such as hemocytes, fat body, trachea, and nerve cells. BV enters these cells via adsorptive endocytosis (Volkman & Goldsmith, 1985). In this second round of replication the BV phenotype is produced, but the majority of production is directed to the occlusion of virions within the polyhedra (Volkman et al., 1976). The virus particles occluded within polyhedra (Polyhedra-derived virus; PDV) obtain their envelope by “de novo” morphogenesis within the nucleus (Stoltz et al., 1973; Granados et al., 1981). PDV are genetically identical to BV (Cochran et al., 1982), but the envelope constitution is different (Volkman, 1983; Braunagel & Summers, 1994).

Baculoviruses produce two phenotypes, which have distinct functions. In nature, the BV phenotype spreads infection within the insect being responsible for cell-to-cell infection, while the PDV phenotype is responsible for disease transmission among individuals (Adams et al., 1977). The PDV also have important roles in virus stability and survival in the environment. Under experimental conditions, PDV have high infectivity per os, but present low infectivity when injected into the hemocoel. On the

other hand, BV present low infectivity per os, and high infectivity when injected (Keddie & Volkman, 1985). As BV and PDV perform specialized functions within the insect host, it is expected that these functions are regulated by different proteins. Some baculovirus structural proteins considered of relevance to this study will be discussed in section 1.4.

Genomic organization

Baculovirus transcription is regulated in a cascade fashion, where activation of each set of genes is dependent upon synthesis of proteins from the previous class of genes (Friesen & Miller, 1986). Therefore, baculovirus genes can be grouped into three phases during the infection process: early, late, and very late genes.

Early genes. Early genes are transcribed before baculovirus DNA replication. Transcription experiments have shown that early viral transcription is sensitive to α -amanitin, and therefore is carried out by host RNA polymerase II, the only α -amanitin-sensitive enzyme (Fuchs et al., 1983). The early phase is subdivided into two functionally defined stages, immediate early and delayed early. The immediate early genes do not require any synthesis of viral protein for their expression, since they can be transcribed by uninfected cells. On the other hand, delayed early genes require synthesis of viral proteins for their transcription. Examples of immediate and delayed early genes are the *IE-1* (Guarino & Summers, 1986) and *egt* genes (O'Reilly & Miller, 1989), respectively.

Late genes. Late genes are transcribed during or after DNA replication. This class of genes is transcribed during a short period of time, usually from 12 to 24 hours

post-infection, and encodes primarily structural proteins. The late and very late genes are transcribed by an α -amanitin resistant RNA polymerase (Gruel et al., 1981; Fuchs et al., 1983; Huh & Weaver, 1990). Fuchs et al. (1983) suggested that one or more early genes code for a virus-specific α -amanitin resistant RNA polymerase or for factors that modify one of the host polymerases. The promoter for both classes of genes presents the highly conserved motif, A/GTAAG (Rohrmann, 1986; Rankin et al., 1988; Ooi et al., 1989). The *p74* (Kuzio et al., 1989) and the *gp41* genes (Ayres et al., 1994; Liu & Maruniak, 1995) are examples of late genes.

Very late genes. These genes are hyperexpressed after activation of the late genes and remain active through the end of the infection cycle. The very late gene products include the polyhedrin protein, which forms the matrix of the occlusion body, and the p10 protein, which probably has a role in polyhedra formation (Vlak et al., 1988). In addition, these genes are not involved in the formation of infectious virus particles. They can be deleted from the virus genome without affecting virion production (Vlak et al., 1988). Therefore, the *polyhedrin* and the *p10* genes have been the central focus for the development of baculovirus expression vectors, since their promoters are very efficient, accounting for approximately 50% of the total cell protein content in the terminal stages of infection (King & Possee, 1992; Richardson, 1995; Shuler et al., 1995).

Structural proteins: Polyhedra-derived phenotype

p25. A class of virus mutants, termed few polyhedra (FP), arises frequently upon serial passage in insect cells. These mutants present an altered plaque morphology and produce few polyhedra in comparison to the wild type (many polyhedra-MP) virus

(Fraser & Hink, 1982; Slavicek et al., 1992; Harrison & Summers, 1995; Slavicek et al., 1995). The occurrence of baculoviruses producing few polyhedra in nature could affect the ability to detect these viruses, since their stability and persistence in the environment is dependent upon occlusion in the polyhedra. A 25 kDa protein is necessary for the MP phenotype (Beames & Summers, 1988), and the deletion of the gene encoding for this protein decreases formation of the polyhedra and virion occlusion (Beames & Summers, 1989), producing the FP (few polyhedra) phenotype. The gene encoding for the 25 kDa protein has been characterized for the AcMPV and GmMNPV (Beames & Summers, 1988), and LdMNPV (Bischoff & Slavicek, 1996).

gp41. Phylogenetic studies have revealed that *gp41* genes are highly conserved with 60% nucleotide homology among four different baculoviruses (Liu & Maruniak, 1995). This gene could potentially be used as target region for the development of a PCR-based technique to detect multiple baculovirus species. Monoclonal antibodies studies indicated that gp41 is present only in occluded viruses; it does not appear to be associated with purified nucleocapsids or BV (Whitford & Faulkner, 1992; Ma et al., 1993). The gp41 protein may be located between the envelope membrane and the capsid of the PDV. Presently, the biological function of gp41 is still unknown, but it may be involved in the occlusion of virions within the polyhedra, or in the infection of host midgut cells. The gene encoding this protein was characterized for several baculoviruses, including AcMNPV (Whitford & Faulkner, 1992; Ayres et al., 1994; Kool et al., 1994), HzNPV (Ma et al., 1993), BmMNPV (Nagamine et al., 1991), SfNPV (Liu & Maruniak, 1995), and AgMNPV (Liu & Maruniak, in preparation).

Polyhedral envelope (PE). Surrounding the baculovirus occlusion bodies is an electron dense envelope (calyx) composed of carbohydrate (Harrap, 1972). The calyx function is unknown, but it probably contributes to the OB stability in the environment. A major PE component is the PE (polyhedron electron-dense envelope) protein, which is phosphorylated and thiolly linked to the carbohydrate in the polyhedron envelope (Minion et al., 1979; Whitt & Manning, 1988). Interruption of the gene coding for the PE protein, produces OBs that are more sensitive to weak alkali conditions than wild-type OBs (Zuidema et al., 1989). Therefore, the occurrence of these mutations in nature could affect the environmental detection of baculoviruses.

p10. The hyperexpressed p10 protein is associated with the formation of fibrous networks in the nucleus and cytoplasm of infected cells, and it is also involved with the PDV phenotype (Van der Wilk et al., 1987). Studies with *p10* deletion mutants have observed distinctive cytopathic effects (cpe) in comparison to cells infected with wild-type virus (Williams et al., 1989). These distinctive effects included intranuclear accumulation of granular structures at sites corresponding to the wild-type fibrillar bodies; lack of association between membranes and occlusion bodies; and abnormal membrane attachment to occlusion bodies. However, occlusion body membranes were found to associate normally with the fibrillar bodies in wild-type infections. In one case, a particular deletion mutant produced fragile occlusion bodies which were fragmented by vigorous washing and sonication (Williams et al., 1989). The *p10* gene may play a role in occlusion body stability, and therefore, the lack of this gene in wild-type or genetically-engineered viruses may jeopardize their environmental detection.

Polyhedrin. Virions of NPVs and GVs are occluded in large protein crystals.

The matrix of the occlusion bodies is composed primarily of a single type of protein, polyhedrin in NPVs and granulin in GVs. Polyhedrins and granulins are related in structure and function. They present two highly specialized functions: 1) formation of a protective crystal around the virus; and 2) resistance to solubilization, except under strong alkaline conditions (Rohrmann, 1986). These properties enable baculoviruses to remain viable for many years outside the insect host. Summers & Smith (1976) compared peptide maps of polyhedrins from AcMNPV, *Rachiplusia ou* (RoMNPV), TnSNPV, TnGV, and SfGV, and observed similarities in the peptide maps, but concluded that each protein was different and distinct. The *polyhedrin* gene is not essential for viral replication in cell culture, and it is the most common insertion site for foreign genes in the baculovirus expression system (for a review see King & Possee, 1992; Richardson, 1995; Shuler et al., 1995). DNA sequences have been determined for the *polyhedrin* genes of many baculovirus species, including AcMNPV (Hofft Van Iddenkinge et al., 1983), *Bombyx mori* MNPV (Iatrou et al., 1985), *Anticarsia gemmatilis* MNPV (Zanotto et al., 1992), *Spodoptera frugiperda* MNPV (Gonzalez et al., 1989), *S. exigua* MNPV (van Strien et al., 1992) *Orgyia pseudotsugata* MNPV (Leisy et al., 1986), *Anagrapha falcifera* MNPV (Federici-personal communication), *Helicoverpa zea* SNPV (Cowan et al., 1994), *Lymantria dispar* NPV (Chang et al., 1989), *Mamestra configurata* NPV (Li et al., 1997), among others. The *polyhedrin* gene is one of the most conserved genes among baculoviruses (Rohrmann, 1992; Zanotto et al., 1993; Liu, 1997). It presents over 80% sequence identity within lepidopteran NPVs, about 50% identity between lepidopteran

NPVs and GVs, and about 40% sequence identity between lepidopteran and a hymenopteran NPV (Rohrmann, 1992). Therefore, the *polyhedrin* gene is a good candidate for the construction of generic primers for PCR amplification, which could be used to detect multiple baculoviruses in the environment.

Regulatory proteins

Several genes code for regulatory proteins. Some of these are immediate early genes such as the *IE-0* and *IE-1*, which represent the only example of spliced genes in baculoviruses (Chisholm & Henner, 1988). The AcMNPV IE-1 regulatory protein stimulates the expression of some baculovirus delayed early promoters, as shown by transient expression (Guarino & Summers, 1986a), and inhibits the expression of other immediate early genes (Carson et al., 1991). Furthermore, *IE-1* also increases the transcription rate of several baculovirus promoters when cis-linked to hr regions (Guarino & Summers, 1986b; Rodems & Friesen, 1995).

Proteins involved in viral DNA replication

An example of protein involved in viral DNA replication is the DNA polymerase, which is a delayed early gene (Kelly & Lescott, 1981; Miller et al., 1981; Kelly, 1982; Tomalski et al., 1988). Additional delayed-early genes such as the *PCNA* (ETL) and the *helicase* gene, also encode proteins involved in baculovirus DNA replication. Both gene products are involved in replication and late gene transcription, and mutation in these genes produce virus with delayed replication and late gene expression (Crawford & Miller, 1988; O'Reilly et al., 1989; Lu & Carstens, 1991).

Homologous repeats

Many baculoviruses have interspersed homologous regions (hrs) along their genome. These regions were first described on the AcMNPV genome (Cochran & Faulkner, 1983), and it was suggested that these homologous regions might play a role in the replication and expression of AcMNPV, because of their conserved nature. Transient expression assays have shown that the AcMNPV hrs enhance expression of the reporter gene, chloramphenicol acetyltransferase (CAT), under the control of viral delayed-early genes (Guarino & Summers, 1986a; Nissen & Friesen, 1989; Rodems & Friesen, 1993); and immediate early genes (Carson et al., 1991; Rodems & Friesen, 1995). However, the AcMNPV hr5 region did not enhance transcription from a late baculovirus promoter or from a host-derived promoter (Rodems & Friesen, 1993). In addition, the homologous regions in the AcMNPV genome function as an origin of virus DNA replication (Kool et al., 1993; Leisy et al., 1995). Similar regions have been identified in other baculoviruses such as, *Choristoneura fumiferana* MNPV (Arif & Doerfler, 1984; Kuzio & Faulkner, 1984; Xie et al., 1995), LdMNPV (McClintock & Dougherty, 1988; Pearson & Rohrmann, 1995), OpMNPV (Theilmann & Stewart, 1992), BmMNPV (Majima et al., 1993), and AgMNPV (Garcia-Maruniak et al., 1996).

The AgMNPV hr4 region has been sequenced and analyzed for two genomic variants, 2D and D7 (Garcia-Maruniak et al., 1996). DNA deletion or duplication events accounted for the difference between these two variants. AgMNPV-2D contained 10 repeat elements of 127 to 128 bp, while AgMNPV-D7 was 381 bp smaller. PCR amplification of the hr4 region of plaque-purified isolates obtained from AgMNPV wild-

type preparations, showed that the isolates differ from each other by their number of 127 bp repeats (Garcia-Maruniak, unpublished). These data indicate that the hr4 region could potentially be used as target for PCR amplification, to distinguish different AgMNPV isolates.

Importance of Baculoviruses

Many insect pests of economic importance are susceptible to baculoviruses including 34 families of Lepidoptera and a few families of Hymenoptera, Diptera, Coleoptera, Neuroptera, Trichoptera, Thysanura, and Siphonaptera (Tanada & Kaya, 1993). For this reason, baculoviruses have been used for decades to control insects, especially lepidopteran pests. Baculoviruses have also been developed as protein expression vectors, being able to express biologically active products (Summers & Smith, 1987; King & Possee, 1992; Tanada & Kaya, 1993; Richardson, 1995; Shuler et al., 1995). These applications make baculoviruses an extremely important group of viruses due to their usefulness in agriculture and in biotechnology.

Use of baculoviruses as microbial control agents

Baculoviruses are widely used to control insects in developed and developing countries, because they present several advantages in comparison to chemical pesticides. Baculoviruses are non-pathogenic to vertebrates and plants (Summers et al., 1975; Payne, 1982; Miller et al., 1983; Miller, 1988); baculoviruses are unable to penetrate the nuclei of mammalian cells (Carbonell & Miller, 1987); they are specific to a few closely related insects, usually within a single family (Ignoffo, 1968); they are compatible with integrated pest management programs (IPM); their use is not accompanied by

undesirable residues in the environment. Furthermore, only one application of baculoviruses is usually enough to control insects (Moscardi, 1989; Fuxa et al., 1993), and to produce yields comparable to those achieved by using chemicals, but at a lower cost (GERATEC, Inc.).

In the United States, several baculoviruses have been registered or are being considered for development as viral insecticides. Currently, the baculoviruses registered for use include SeMNPV, HzSNPV, AcMNPV, AfMNPV, *Cydia pomonella* (codling moth) GV (Mark Beach, formerly Biosys Inc., personal communication, 1996), LdMNPV, and *Neodiprion sertifer* SNPV (U. S. Forest Service, USDA).

Several baculoviruses are used to control forest pests, such as the spruce sawfly *N. sertifer* (Bird & Whalen, 1953), the gypsy moth *L. dispar* (Lewis, 1981; Podgwaite & Mazzone, 1981), and the Douglas-fir tussock moth *O. pseudotsugata* (Hughes & Addison, 1970; Martignoni, 1978). Baculoviruses are also being used to control insect pests in orchards. A good example is the use of a granulosis virus to control the codling moth, *C. pomonella*, which is a serious problem in apple and pear orchards (Huber, 1982; Tanada, 1964). In annual crops, a classical example is the use of the *Heliothis* NPV to control *Heliothis* species in cotton, corn, sorghum, soybeans, tobacco, and tomato (Ignoffo & Couch, 1981). This virus was the first viral insecticide to be registered in the U.S. and its industrialization and commercialization began in 1961 (Ignoffo, 1973).

Baculoviruses have great potential for use in developing countries because regulatory requirements are usually less costly and complex, and labor is abundant and relatively cheap. In Brazil, the use of the *Anticarsia gemmatilis* NPV to control the

velvetbean caterpillar in soybeans represents one of the most successful examples in the world of baculoviruses used as microbial control agents. The virus was isolated from infected velvetbean caterpillars in Campinas, Brazil, in 1972 (Allen & Knell, 1977), and it was subsequently found in other regions (Carner & Turnipseed, 1977). Preliminary research showed that this NPV was highly virulent to *A. gemmatilis* larvae (Carner & Turnipseed, 1977; Moscardi et al., 1981). In 1979, the National Center for Soybean Research (EMBRAPA-CNPSo) started a program in conjunction with the extension service to develop this microbial insecticide and to educate soybean growers about its use. Currently, the AgMNPV is applied to more than 1 million hectares of soybeans annually in Brazil (Moscardi & Sosa-Gomez, 1992). Field trials in Florida, U.S.A., have shown that only one application of AgMNPV efficiently controls velvetbean caterpillar populations in soybeans (Funderburk et al., 1992). Therefore, the AgMNPV has good potential for use in soybean integrated pest management in the southeastern United States.

However, the use of baculoviruses as microbial control agents has been restricted to agricultural or forestry systems that can tolerate some damage without economic loss (Bonning & Hammock, 1992), because of the extensive time that these viruses take to kill their insect hosts (usually 4 to 14 days). In addition, the high specificity of baculoviruses is frequently seen as a disadvantage by growers and industry. Growers usually prefer to use a single product to solve their insect problems, while the industry needs to sell a product to be used over large areas, or to control several different insects, in order to

quickly pay off costs associated with research, manufacturing, and registration of the product.

The use of recombinant DNA techniques to genetically improve baculoviruses is an attractive alternative to expand their use as insect control agents. Several research groups have attempted to genetically engineer baculoviruses, with the main goal being to decrease the time needed for the virus to kill the insect host. The status of research on genetically-engineered baculoviruses and aspects concerning their release in the environment will be discussed in item 3.0.

Use of baculoviruses as expression vectors

The potential of baculoviruses as expression vectors was recognized because of some of their features. Some of these characteristics include their extendable rod-shaped capsid; the existence of a group of genes that are replaceable, because they are not essential for synthesis of nonoccluded viruses; the availability of strong promoters; the nonsusceptibility of mammalian cells to baculoviruses; the limited host range of baculoviruses, among others (Miller, 1981).

The first expression vector utilized the AcMNPV *polyhedrin* promoter, which is highly expressed and regulated. The *polyhedrin* gene was modified for the insertion of foreign genes, allowing the expression of prokaryotic or eukaryotic recombinant proteins (Smith et al., 1983). To date, most transfer vectors contain AcMNPV sequences including the *polyhedrin* promoter and varying amounts of DNA sequences flanking the *polyhedrin* gene. The vector contains bacterial plasmid genes and the foreign gene is transferred back to wild-type AcMNPV by homologous recombination within insect

cells, which are transfected with the plasmid vector and the wild-type virus (Luckow & Summers, 1988).

The baculovirus expression system has become highly useful because it has the ability to express high levels of protein, the posttranslational modifications are similar to eucaryotic systems, and the result is the production of biologically active products. To date, baculovirus vectors have been used for the expression of a wide variety of genes to produce insecticidal products, DNA binding proteins, viral structural proteins, proteins for pharmaceutical purposes, and other proteins for biological studies (King & Possee, 1992; Richardson, 1995; Shuler et al., 1995).

Genetically-engineered Baculoviruses

One of the first attempts to genetically engineer baculoviruses was made by Carbonell et al. (1988), who inserted a synthetic insect neurotoxin gene (*BelI*) of the scorpion *Buthus eupeus* into the AcMNPV genome. The authors observed substantial expression of a polyhedrin/toxin fusion gene, but paralytic activity was not detected. Positive activity was obtained by inserting another scorpion toxin gene (*AaII*) from the scorpion *Androctonus australis* into the BmNPV genome under the control of the *polyhedrin* gene promoter (Maeda et al., 1991). *B. mori* larvae infected with this recombinant virus stopped feeding by 45-55 hours post infection (hr p. i.), and all larvae died by 60 hr p.i., representing a 40% increase in the speed of kill when compared to a wild type BmNPV. The *AaII* toxin gene was also inserted into the AcNPV genome under the control of the *p10* promoter (McCutchen et al., 1991). Bioassays with the

recombinant virus on 2nd instar *Heliothis zea* larvae showed a significant decrease in the time to kill (LT_{50} = 88 hours) compared to wild-type AcNPV (LT_{50} = 125 hours).

Insecticidal crystal protein (ICPs) genes of *Bacillus thuringiensis* in its full-length, truncated, and mature forms have also been engineered into the AcMNPV genome under the control of either the *polyhedrin* or the *p10* promoter (Martens et al., 1990; Merryweather et al., 1990; Ribeiro & Crook, 1993; Martens et al., 1995). Although very large amounts of active ICPs were produced in these experiments, they neither improved the virulence of AcNPV (Merryweather et al., 1990) nor decreased the time to kill the insect larvae (Ribeiro & Crook, 1993; Martens et al., 1995).

A toxin from the straw itch mite *Pyemotes tritici* has also been used to construct a recombinant AcMNPV with improved insecticidal activity (Tomalski & Miller, 1991). Although the mode of action of the mite toxin TxP1 is still unknown, the introduction of its cDNA into the AcMNPV genome reduced time to kill by 30-40% in comparison to the wild-type virus. Paralysis was observed within two days after larval injection with the recombinant virus.

The introduction of sequences coding for insect hormones or enzymes involved in some regulatory aspect of the insect's endocrine system is another alternative to improve baculoviruses as microbial insecticides. Examples are the introduction of the diuretic hormone from *Manduca sexta* into the BmMNPV genome (Maeda, 1989) and the introduction of a juvenile hormone esterase (JHE) into the AcMNPV genome (Hammock et al., 1990; Bonning et al., 1992; Eldridge et al., 1992; Bonning et al., 1994).

The deletion of some viral encoded genes might improve the insecticidal properties of baculoviruses. One example is the *egt* gene, which encodes an ecdysteroid UDPglucosyltransferase and catalyzes the transfer of glucose from UDPglucose to ecdysteroids (insect molting hormones). This viral gene has been characterized for the AcMNPV (O'Reilly & Miller, 1990) and LdMNPV (Riegel et al., 1994). Further studies with the AcMNPV *egt* gene demonstrated that expression of this gene in wild-type viruses prolongs the length of time that the insect feeds after infection, by inhibiting molting of the infected host (O'Reilly & Miller, 1991). In contrast, it was observed that *S. frugiperda* larvae infected with AcMNPV *egt* deletion mutants fed for a shorter period of time and died earlier when compared to wild-type AcMNPV.

The first field trials of genetically engineered baculoviruses were completely contained and involved a genetically marked AcNPV (Bishop, 1986); a crippled and tagged virus which did not produce polyhedrin; and a recombinant virus encoding a non-functional gene (Bishop, 1989). Wood et al. (1994) conducted field trials involving the release of a polyhedrin-negative AcNPV which was co-occluded with wild-type virus. Monitoring of the field site in subsequent years demonstrated that the polyhedrin negative virus disappeared rapidly. Subsequently, a recombinant AcNPV expressing the insect selective toxin, AaHIT, from the scorpion *A. australis* was released in field trials in the United Kingdom (Cory et al., 1994). Insects infected with the recombinant virus consumed about 25% less than the equivalent wild-type treatment, and there was earlier death of recombinant-infected insects.

The use in the environment of genetically engineered organisms, including baculoviruses, must be handled systematically and cautiously, taking into consideration the benefits and the risks. Risk assessment has been defined as "the process of obtaining quantitative or qualitative measures or risk levels, including estimates of possible health effects and other consequences as well as the degree of uncertainty in those estimates" (Fiksel & Covello, 1986). According to Levin (1982), the possibility of harm in the environment is the product of six components: release, survival, multiplication, dissemination, transfer of genetic information, and harm. The release of a recombinant baculovirus should be preceded by extensive studies of the ecology of both the wild-type and the recombinant virus. These studies should be undertaken in the laboratory and in confined environments. Thus, the ability to detect and to distinguish between wild-type and recombinant viruses becomes extremely important. The development of a sensitive and rapid detection technique which can target conserved and variable DNA regions of the organisms being evaluated would be instrumental in the study of the ecology, and environmental fate of these organisms. In addition, this technique could be used in quality control of programs in which baculoviruses (wild-type or recombinant) are being applied to control insects.

Ecology of Baculoviruses

According to Fuxa & Tanada (1987), the definition of "epizootic" in insect pathology is the same as used to describe "epidemic" in medical epidemiology. Both are defined as an unusually large number of cases of disease in a host population. The definition of "unusually large numbers" varies according to the pathogen and must be

based on the study of each disease for a number of years. Development of natural baculovirus epizootics is dependent upon the strain virulence, the capacity of the virus to persist, and to disperse in the environment. Natural epizootics of baculoviruses have been observed for Lepidoptera and Hymenoptera NPVs (Entwistle & Evans, 1985).

Persistence

The quantity of virus persisting between insect host generations and initiating new infections is a function of a complex interaction between the virus, the host insect, and the environment (Evans & Harrap, 1982). Evans & Harrap (1982) have correlated plant permanency and complexity of structures with long-term persistence of virus outside the living host. The authors pointed out that the presence of foliage throughout the year provides long-term retention of the virus on the plant itself. This is frequently the case with baculoviruses infecting forest pests, such as the NPV of *Gilpinia hercyniae* on spruce foliage in Wales (Entwistle & Adams, 1977), the LdNPV which was able to overwinter in the bark of oak and red maple (Podgwaite et al., 1979), and the NsNPV which persisted for 21 months in pine foliage (Mohamed et al., 1982). In contrast, viruses are rapidly inactivated on the foliage of annual crops due to the temporary nature of the plant itself. Jaques (1975) concluded that deposits of NPV and GV on the foliage of annual crops retain activity for less than two weeks, and are mainly inactivated by the ultraviolet component of the sunlight. Studies in the field and in the laboratory have demonstrated that temperature and rain are not main factors in baculovirus inactivation (David & Gardiner, 1966; David & Gardiner, 1967a; David & Gardiner, 1967b; Gudauskas & Canerday, 1968). In contrast, ultraviolet light inactivates baculoviruses

rapidly. For example, HznPV was totally inactivated by a 5 min exposure to ultraviolet at 2 inches from the source (Gudauskas & Canerday, 1968). In the field, the granulosis virus of *Pieris brassicae* was greatly inactivated after 3 hr of direct exposure to sunlight on the upper surface of cabbage leaves (David et al., 1968). Jaques (1975) showed that TnNPV and *P. rapae* GV lost about 50% of their activity 2 days after application when exposed to direct sunlight on leaves of cabbage. The persistence of HznPV, *Heliothis armigera* NPV, AcMNPV, and AgMNPV on heading grain sorghum as a control for the corn earworm declined to low levels at four days after application (Young & McNew, 1994).

The soil is a long term reservoir for baculoviruses in the environment, regardless of the life style of the host, type of plant on which it feeds, or mode of action of the virus (Evans & Harrap, 1982; Entwistle & Evans, 1985). Baculoviruses adsorb strongly to the soil and remain in high concentrations near the soil surface (Evans & Harrap, 1982). In one of the early studies, the viral activity of soil treated with polyhedra of TnNPV remained around 25% of the original activity more than five years after treatment (Jaques, 1967). Furthermore, application of polyhedra to soil under plants caused more prolonged viral contamination of foliage than direct application to the foliage. This study also correlated rainfall and viral contamination of foliage. David & Gardiner (1967b) found that *P. brassicae* GV lost little activity in soil or sand in two years. *Pseudoplusia includens* NPV applied at 247 Larval Equivalents/ha initiated epizootics in soybean looper populations in soybean fields, and it persisted in the soil at high concentrations from two weeks after application throughout the following season (McLeod et al., 1982).

Laboratory bioassays of aqueous soil suspensions from pine plots treated one year and treated for two consecutive years have shown that the NPV of *N. sertifer* persisted in the soil for 21 months, causing 11 and 20% mortality in test larvae, respectively (Mohamed et al., 1982).

Dispersal

Baculovirus dispersion occurs through abiotic and biotic agents. Abiotic spread occurs mainly by the action of wind and rain. For example, Thompson & Scott (1979) suggested that the onset of natural epizootics of NPV disease in *Orgyia pseudotsugata* after periods of low host density is by wind-borne soil containing polyhedra which have survived for several years. Jaques (1967) showed in field and laboratory tests that foliage of cruciferous plants grown in soil treated with TnNPV polyhedra were contaminated with the virus, probably due to virus-contaminated soil being splashed onto foliage by rain.

Besides being dispersed by the host and secondary hosts, baculoviruses are also dispersed by other biotic agents such as mammals, birds, arthropod predators, and parasitoids. In this review I will address mainly the role of insect predators as agents of baculovirus dispersion. Several authors have suggested that predators are important agents in baculovirus dispersal (Hostetter, 1971; Capinera & Barbosa, 1975; Smirnov, 1975; Beekman, 1980; Cooper, 1981). Other studies have shown that predatory insects including carabid beetles (Capinera & Barbosa, 1975; Vasconcelos et al., 1996); sarcophagid flies (Hostetter, 1971); and hemipterans (Beekman, 1980; Cooper, 1981; Abbas & Boucias, 1984; Young & Yearian, 1987) excrete infective NPV for several days

after feeding on infected larvae. Therefore, the most probable mechanism of baculovirus dispersal by predators is feeding on NPV-contaminated larvae and subsequent release of viable polyhedra through the feces. External contamination of predators could be another mechanism for baculovirus dispersion. However, results in the literature indicate otherwise. For example, the highest percentage of predators contaminated by AgMNPV were observed 7 and 10 days after baculovirus application in soybean field studies conducted by Fuxa et al. (1993) and Moraes et al. (manuscript in preparation), respectively. The authors suggested that if predators became contaminated due to baculovirus spraying or due to contact with contaminated vegetation, the highest percentages of NPV contamination in predators would have occurred immediately after virus application.

Baculovirus dispersal by predators is dependent on the acceptance of infected larvae by predators as a food source. Some predators have shown no discrimination between healthy and diseased larvae. For example, *Podisus maculiventris* (Say) accepted both infected and healthy *A. gemmatilis* larvae with no distinction (Abbas & Boucias, 1984). In contrast, *Nabis roseipennis* Reuter showed a preference for diseased *A. gemmatilis* larvae when given a choice between healthy and diseased prey (Young & Yearian, 1989; Young & Kring, 1991). Currently, numerous studies have implicated predators as agents of baculovirus dispersion, but very few have actually proven this fact. Field experiments with carabid beetles which had previously fed on NPV-diseased *Mamestra brassicae* larvae showed that sufficient virus was transferred from the predator feces to the soil, causing low levels of mortality in *M. brassicae* larval populations

(Vasconcelos et al., 1996). Larval mortality was observed when uninfected *A. gemmatilis* larvae were caged for 4 days on soybean plants with *N. roseipennis* adults that had fed on NPV-infected larvae (Young & Yearian, 1987). Parasitoids increase virus dispersal in a similar way to predators, but generally the dispersal mechanism is by external contamination rather than gut passage. A review by Evans (1986) discusses several examples of baculovirus dispersion by parasitoids.

Transmission

Transmission of baculoviruses is related to all aspects of their ecology and encompasses virus dispersion and persistence, which were discussed above. In a more strict sense, transmission can be divided into vertical and horizontal. Viruses can be passed to newly hatched host larvae by means of vertical transmission. Evans (1986), in his review of baculovirus ecology and epizootiology, mentions several examples of successful transmission of baculoviruses via the adult stage. It is important to note, however, that none of those examples were due to transovarium transmission by incorporation in the egg. According to Evans (1986), true transovum transmission appears to be confined to species having gut infections in the adult stage, such as the hymenopteran sawflies, and vertical transmission by adult Lepidoptera is probably due to external contamination. This author points out that vertical transmission in baculoviruses operates independently of host threshold density since virus is passed directly to progeny. Therefore, vertical transmission could be an important mechanism for maintenance of virus when host populations are low.

According to Entwistle & Evans (1985), epizootics of virus diseases in agricultural pests rely heavily, especially for their initiation, on the soil and the host as a reservoir. Therefore, the more host generations in a growing season, the better chances are of epizootic development. Young et al. (1987) studied the transmission of the *A. gemmatilis* NPV in uniform- and mixed-aged populations of velvetbean caterpillar on field-caged soybeans. The authors observed a low level of NPV transmission from primary infected to uninfected larvae in a uniform-aged velvetbean caterpillar population. In contrast, virus transmission in mixed-age populations was greater than in uniform-aged populations, especially when primary infected larvae were of the same age structure as the uninfected population. The authors observed that the early presence of disease in the host population results in an early death of primary inoculum larvae (PIL), therefore, releasing inoculum for secondary transmission while many of the uninfected host population are still small and more susceptible to the virus. These results reinforce the importance of timing baculovirus treatments against small larvae to optimize population reduction from primary and secondary inoculum.

Detection of Baculoviruses

Baculoviruses routinely can be detected by counting stained or unstained polyhedra preparations under the light microscope (Kaupp & Burke, 1984; Travermer & Connor, 1992). The detection limit for this method is 1×10^6 PIBs per milliliter, and the earliest detection is generally about 2 to 4 days post-infection at 22°C (Entwistle & Evans, 1985; Evans, 1986). Microscopic diagnosis can be time consuming if large number of samples are to be examined.

Bioassays are also routinely used to assess the incidence of baculovirus disease under field conditions. Bioassays are usually very sensitive and can be used to detect the presence of the virus on the foliage, in soil (Hukuhara, 1975; Fuxa et al., 1985; Fuxa & Richter, 1994; Wood et al., 1994), predators (Boucias et al., 1987; Young & Yearian, 1990; Fuxa et al., 1993), and predator feces (Abbas & Boucias, 1984; Young & Yearian, 1987; Vasconcelos et al., 1996). Host mortality in bioassays is a direct measure of virus viability, however, virus identification is only tentative, and is based on susceptibility of a particular host species. To identify the viral species with certainty other methods must be employed, such as restriction enzyme profiles (Wood et al., 1994), Southern blots, or PCR amplification (Moraes & Maruniak, 1997). Furthermore, bioassays are tedious and time consuming (Longworth & Carey, 1980), because it usually takes from two to 14 days to assess total host mortality.

Baculoviruses can be detected serologically using antisera raised to polyhedra, virions, and virion proteins. Fluorescent antibody radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) are the most employed methods. The use of ELISA to detect virus particle antigens enables baculovirus detection earlier and at a higher level of sensitivity than polyhedra counts (Evans, 1986). Furthermore, serological methods are extremely useful for detecting baculoviruses that cannot be measured by bioassay due to difficulties in rearing the host insect (Crawford et al., 1978; Webb & Shelton, 1990). However, serological techniques present some problems limiting their use: 1) all the polyhedrin proteins seem to cross-react (Smith & Summers, 1981); 2) it is difficult to obtain virion preparations free of polyhedrin; 3) polyhedrin might contain at

least some common antigenic determinants (Wood, 1980); and 4) the presence of host extracts usually decreases the sensitivity of baculovirus detection (Crook & Payne, 1980; Longworth & Carey, 1980). Some authors have overcome cross-reaction problems by using monoclonal antibodies (Naser & Miltenburger, 1982, 1983; Quant et al., 1984) or by choosing a more specific method such as the double antibody sandwich (Crook & Payne, 1979). Nonetheless, there are many reports in the literature on the use of serological methods to detect and to identify baculoviruses (Crook & Payne, 1979; Longworth & Carey, 1980; Naser & Miltenburger, 1982, 1983; Webb & Shelton, 1990), as well as to study their relatedness (McCarthy & Lambiase, 1979; Brown et al., 1982; Knell et al., 1983).

Radioimmunoassay (RIA) techniques also have been used to detect and to identify baculoviruses (Crawford et al., 1977, 1978; Ohba et al., 1977; Smith & Summers, 1981). However, radioimmunoassay techniques use radioisotopes (^{125}I), which require careful and more specialized handling. Other techniques have sporadically been used to detect baculoviruses, including single radial diffusion (Scott et al., 1976), immunoperoxidase assay (Summers et al., 1978), serum neutralization (Martignoni et al., 1980), and dipstick immunoassay (Nataraju et al., 1994).

DNA techniques such as dot blot hybridization assays have also been developed to detect baculoviruses in host larvae at levels between 1.0 and 0.1 ng of NPV DNA (Ward et al., 1987; Keating et al., 1989; Kukan & Myers, 1995). These DNA hybridization techniques are specific and sensitive but, as with radioimmunoassay techniques, they also utilize radioactive materials which restrict their use. Kaupp &

Ebling (1993) have developed horseradish peroxidase-labeled DNA probes and enhanced chemiluminescence procedures to detect baculoviruses in gypsy moth (*L. dispar*) and eastern spruce budworm larvae (*C. fumiferana*). This non-radioactive technique presented detection levels similar to those observed for ^{32}P -labeled probes. However, cross-reactions were observed between LdNPV DNA probes and CfNPV.

The polymerase chain reaction (PCR) is a rapid and inexpensive technique which does not require radioactive materials, and it produces large amounts of DNA from very small amounts of starting material (McPherson et al., 1993). The PCR technique is based on the principle that DNA polymerases carry out the in vitro synthesis of complementary DNA in the 5' to 3' direction using a single-stranded template. During PCR, two primers are used, each complementary to opposite strands of the DNA target region. The DNA template is denatured by heating, and the primers are arranged so that each primer extension reaction directs synthesis of DNA towards the other. Therefore, primer 1 directs synthesis of a strand of DNA which can then be primed by primer 2 and vice-versa. The result is de novo synthesis of the region of DNA flanked by the two primers (Taylor, 1993). PCR has been largely used as a detection and diagnosis tool for many animal (Wiedmann et al., 1993), human (Abbaszadegan et al., 1993; Ansari et al., 1992; Ramelow et al., 1993; Rousell et al., 1993; Lees et al., 1994), and plant viruses (Mehta et al., 1994; Thomsom & Dietzgen, 1995; Munford et al., 1996). The PCR technique has enabled detection of microorganisms from samples with relatively poor quality, containing high levels of inhibitors, such as shellfish, wastewater, groundwater, soil, fecal extracts, and semen, among others (Ansari et al., 1992; Abbaszadegan et al., 1993;

Rousell et al., 1993; Wiedmann et al., 1993; Lees et al., 1994). Furthermore, detection of pathogens by PCR is highly sensitive. For example, Ansari et al. (1992) were able to detect as little as 0.04 pg of HIV-1 target DNA; Abbaszadegan et al. (1993) developed a PCR assay which detected 10^{-1} plaque-forming units (PFU) of poliovirus type 1; and Lees et al. (1994) detected less than 10 PFU of poliovirus in shellfish. PCR has also been used to detect baculoviruses, although under very specific conditions. Webb et al. (1991) used PCR to screen less than 10 ng of starting viral template from baculovirus expression vector recombinants in cell cultures. Burand et al. (1992) detected baculovirus DNA sequences from viral occlusion bodies (OB) contaminating the surface of gypsy moth eggs.

Present Study

In this study, the *polyhedrin* gene was chosen as target template for PCR amplification, because of its highly conserved nature (Rohrmann, 1992; Zannotto, 1993). PCR for the *polyhedrin* gene was optimized and enabled detection of eight different species of baculoviruses, which were distinguished by restriction enzyme analysis (Moraes & Maruniak, 1997). Subsequently, the PCR technique was used to detect AgMNPV from environmental samples including larval hosts, insect predators, and soil. The technique was expanded to detect different AgMNPV genotypes. To accomplish this goal a highly variable region, hr4, of the AgMNPV genome was chosen. PCR amplification of the hr4 region distinguished two different AgMNPV genotypes applied in soybean fields, by the different size of the amplified products. A methodology to extract baculovirus DNA from soil was developed and produced DNA suitable for PCR

amplification. This was the first report on the direct extraction of baculovirus DNA from soil.

This study will provide a specific, fast, and sensitive way to detect and to identify baculoviruses in the environment. This technique which targets conserved and variable DNA regions of the viral genome and enables detection at the species and genotypic level, will benefit the study of the environmental fate and ecology of baculoviruses. Furthermore, these procedures will be useful in quality control of programs in which baculoviruses (wild-type or recombinant) are being applied to control insects as well as in quality control of the commercial production of these viruses.

CHAPTER 2 DETECTION AND IDENTIFICATION OF MULTIPLE BACULOVIRUSES

Introduction

Baculoviruses are enveloped, double stranded DNA viruses with circular genomes that comprise the largest and most widely studied group of viruses pathogenic to insects (Federici, 1986). Baculoviruses have great potential to be used as biological insecticides. In the United States eight baculoviruses have been registered for commercial use with the Environmental Protection Agency (Mark Beach, former Biosys Inc., personal communication, 1996). However, the use of these products on a large scale has been limited mainly due to the extensive time required to kill the insect host. Nevertheless, in Brazil *Anticarsia gemmatilis* multiple-embedded nuclear polyhedrosis virus-AgMNPV (Baculoviridae: Nucleopolyhedrovirus) has been applied to about one million hectares of soybean annually, for several seasons, for control of the velvetbean caterpillar (*A. gemmatilis* Hübner) (Moscardi, 1989; Moscardi & Sosa-Gomez, 1993).

Attempts to decrease the time mortality of baculoviruses have been made primarily through genetic engineering. Examples are the insertion of the *Bacillus thuringiensis delta-endotoxin* gene into the AcMNPV genome (Martens et al., 1990; Merryweather et al., 1990); the insertion of insect specific scorpion toxins (Carbonell et al., 1988; Maeda et al., 1991; McCutchen et al., 1991; Stewart et al., 1991); and the use of

insect hormones or enzymes which can affect the insect's endocrine system (Maeda, 1989; Eldridge et al., 1991). The commercialization and release of recombinant viruses in the environment creates the concern that they might cause ecological disturbances, such as the displacement of native microorganisms, adverse effects in non-target organisms, and horizontal transfer of DNA into non-target organisms (Leung et al., 1994). An improved nuclear polyhedrosis virus of the alfalfa looper (AcMNPV) has been already released in field trials in the United Kingdom, and it kills the larval host at a faster rate when compared to the wild type virus (Cory et al., 1994). The sensitive and rapid detection of wild type and recombinant viruses in the environment and in insects, both host and nonhost, is fundamentally important. It can potentially contribute to monitor and to study the environmental fate of released recombinant organisms as well as to better understand the ecology and epizootiology of wild type baculoviruses, improving their efficiency as biological control agents. Several methods have been employed to detect baculoviruses, such as microscopic diagnosis (Kaupp & Burke, 1984; Traverner & Connor, 1992), serological techniques (Crook & Payne, 1980; Langridge et al., 1981; Brown et al., 1982; Naser & Miltenburger, 1982; Naser & Miltenburger, 1983; Webb & Shelton, 1990), radioimmunoassay techniques (Ohba et al., 1977; Smith & Summers, 1981; Knell et al., 1983), and DNA dot blot hybridization assays (Ward et al., 1987; Keating et al., 1989). Nonetheless, the use of these techniques has been limited because they are either tedious and unreliable (microscope examination), or, as with serological methods, cross reactions with other species of NPVs may occur (McCarthy & Henchal,

1983), or because they utilize radioactive materials (DNA hybridization techniques and radioimmunoassay).

The polymerase chain reaction (PCR) is a highly sensitive technique which amplifies target DNA sequences and does not employ radioactive material. PCR has been used extensively to detect many animal and human pathogens such as enteric adenoviruses (Roussel et al., 1993), human and bovine herpesviruses (Borchers & Slater, 1993; Wiedmann et al., 1993), influenza virus (Zuckerman et al., 1993), and bluetongue virus (Wilson & Chase, 1993), among others. Webb et al. (1991) reported the use of PCR to screen baculovirus expression vector recombinants in cell cultures. They were able to detect the amplified gene starting with less than 10 ng of viral template DNA. Burand et al. (1992) were able to detect baculovirus DNA sequences from viral occlusion bodies (OB) contaminating the surface of gypsy moth eggs.

The objective of this research was to develop a technique using the polymerase chain reaction and restriction enzyme analysis to detect multiple baculoviruses in their respective insect hosts, non target insects, and in the environment. To accomplish this, a highly conserved DNA sequence within the coding region of the *polyhedrin* gene was chosen. The *polyhedrin* gene codes for the occlusion body protein, polyhedrin, which has approximately 80% sequence homology among the NPVs and over 50% similarity among lepidopteran NPVs and GVs (Rohrmann, 1986). The baculoviruses evaluated in this study are either being currently commercialized or have good potential to be used as microbial control agents.

Materials and Methods

Viral DNA

The baculoviruses tested and typed in this study were *Autographa californica* multiple-embedded nuclear polyhedrosis virus (AcMNPV), *A. gemmatilis* MNPV (AgMNPV), *Bombyx mori* MNPV (BmMNPV), *Orgyia pseudotsugata* MNPV (OpMNPV), *Spodoptera frugiperda* MNPV (SfMNPV), *S. exigua* MNPV (SeMNPV), *Anagrapha falcifera* MNPV (AfMNPV), and *Heliothis zea* single-embedded nuclear polyhedrosis virus (HzSNPV). Viral DNAs from these eight viruses were extracted by successive phenol and ether extractions (Maruniak et al., 1986; Appendices A-C) for use as templates in PCR reactions. The sources of viral DNA were the following infected larvae; *Spodoptera frugiperda* (SfMNPV), *S. exigua* (SeMNPV), *A. gemmatilis* (AgMNPV), *Trichoplusia ni* (AcMNPV); infected *Spodoptera frugiperda* cells -SF9, with a MOI of 1 (SfMNPV, SeMNPV, AgMNPV, and AcMNPV); and commercial formulations (AfMNPV and HzSNPV, provided by Dr. R. Bell, USDA-ARS-SIML). BmMNPV DNA and OpMNPV polyhedra were provided by Drs. S. Maeda and G. Rohrmann, respectively).

Primer Design

The coding region of the *polyhedrin* gene, which is highly conserved among NPVs, was targeted as template DNA. The DNA sequence for this gene has been previously determined for each of the baculoviruses tested: AcMNPV (Hooft Van Iddekinge et al., 1983), BmMNPV (Iatrou et al., 1985), OpMNPV (Leisy et al., 1986),

AgMNPV (Zanotto et al., 1992), SfMNPV (Gonzalez et al., 1989), SeMNPV (van Strien et al., 1992), AfMNPV (Federici-personal communication), HzSNPV (Cowan et al., 1994). The *polyhedrin* gene sequences were analyzed by the Genetic Computer Group-GCG (Devereaux et al., 1984) in order to obtain a consensus sequence.

Two sets of degenerate primers common for the whole group were designed using the Oligonucleotide Selection Program (OSP) (Hillier & Green, 1991). The DNA sequence for the first set of primers was: 5'TA(CT)GTGTA(CT)GA(CT)AAACAAG 3' (forward) and 5'TTGTA(GA)AAGTT(CT)TCCCAG 3' (reverse), corresponding on the AcMNPV genome, to bases +40 to +57 on the coding strand and bases +614 to +597 on the opposite strand. The second set of primers was a modification of the first one, and its sequence is presented in Table 2.1. The primers were synthesized by the DNA Core Facility of the Interdisciplinary Center for Biotechnology Research at the University of Florida, Gainesville, FL.

PCR Conditions

PCR was optimized as a set of separate reactions for each virus in order to avoid contamination. The conditions which yielded positive amplification or the best amplification yield, were tested two or three times to assure consistency of the results. A negative control, containing all the PCR reagents except the DNA-template, was always included. The PCR reactions were performed in 25 µl containing 200 µM of each dNTP; 5, 12.5, or 20 pmoles of each primer; 1.5, 2.5, or 5.0 mM MgCl₂; 0.5 U of PrimeZyme (Biometra, Inc., Tampa, FL) or 0.8 U of Taq DNA Polymerase (Boehringer Mannheim, Inc., Indianapolis, IN) in 1x reaction buffer for the corresponding enzyme. DNA-

template concentrations varied from 0.5 to 10 ng (Appendix D). Each reaction tube was covered with 25 μ l of mineral oil to prevent evaporation. Amplifications were performed in a PTC-100 Programmable Thermal Cycler (MJ Research, Inc., Watertown, MA), with the amplification cycles consisting of an initial denaturation step of 95°C/1 min, followed by 35 cycles of 94°C/1 min, 48°C/1 min 10 s (annealing), 72°C/1 min 30 s (extension). The final extension step was 15 min. The amplification products were analyzed by 0.7% agarose (FMC, Inc., Rockland, ME) gel electrophoresis in TAE (0.04 M Tris-acetate, 0.001 M EDTA pH 8.0) buffer, stained with ethidium bromide.

Restriction Enzyme Analysis

A number of restriction enzymes were used to fingerprint the different viral PCR products. A 10 μ l aliquot of the respective PCR products was directly used in the restriction enzyme digestions, which were performed according to the manufacturers. Restriction profiles were analyzed in a 3:1 Nusieve GTG:Seakem LE Agarose (FMC) gel electrophoresis in TAE buffer. Gels were stained with ethidium bromide and visualized by UV light.

Results

Two sets of PCR primers were designed from a *polyhedrin* gene consensus sequence based on the sequence analysis of the eight baculoviruses evaluated. The first set of primers successfully amplified DNA from the AcMNPV, AgMNPV, SfMNPV and SeMNPV polyhedrin regions. However, no amplification or non-specific products were observed when the BmMNPV, OpMNPV, AfMNPV and HzSNPV were tested (Data not

shown). This was mainly attributed to the presence of mismatches at the 3' ends of both primers for those particular viruses. The original set of primers was modified by creating a degenerate position at the mismatched base-pairs and by adding one (forward primer) or two (reverse primer) base-pairs at the 3' end. This second set of primers (modified primers) annealed to DNA sequences within the coding region of the *polyhedrin* genes, and yielded an amplified product of 575 base pairs for all eight different baculoviruses. The primer sequences and degree of similarity with the respective viral DNAs are shown in Table 2.1.

The optimal concentration of primers was 12.5 pmoles per reaction. Primer-dimer formation was observed when a higher concentration of primers was used (20 pmole/primer/reaction), whereas a reduced yield of PCR product was observed when a lower concentration was tested (5 pmoles). Concentration of viral DNA did not appear to affect product yield nor specificity in the same degree observed for the primer concentration. Usually, 0.5 to 10 ng of viral DNA per reaction produced good results. In a preliminary experiment, we obtained positive amplification when using as low as 1 pg of AgMNPV DNA (data not shown). Magnesium ($MgCl_2$) concentration was of paramount importance for the PCR reaction. Concentrations of 1.5 mM resulted in no amplification for most of the viruses or resulted in a low PCR product yield; 2.5 mM of $MgCl_2$ produced very specific products, while 5.0 mM, produced non-specific products. PCR was a specific detection method. Positive amplification occurred with DNA extracted from SF9 cells infected with AcMNPV, AgMNPV, SfMNPV and SeMNPV, but did not occur when the cells were uninfected (Figure 2.1).

Table 2.1: Oligonucleotide primers sequence and sequence similarity of the respective viral DNAs.

JM34 (forward)	5' TA(CT)	GTG	TA(CT)	GA(CT)	AAC	AA(GA)	T 3'
AcMNPV	--C	---	--C	--C	---	--G	-
AgMNPV	--T	---	--T	--T	---	--G	-
BmMNPV	--C	---	--C	--C	-- T	--A	-
SfMNPV	--C	---	--C	--C	---	--G	-
SeMNPV	--C	---	--C	--C	---	--A	-
OpMNPV	--C	---	--C	--C	---	--A	-
HzMNPV	--T	---	--C	--C	---	--A	-
AfMNPV	--C	---	--C	--C	---	--A	-
JM33 (reverse)	5'TTG	TA(GA)	AAG	TT(CT)	TCC	CA(AG)	AT3'
AcMNPV	---	--G	---	--C	---	--G	--
AgMNPV	---	--A	---	--T	---	--G	--
BmMNPV	---	--G	---	--C	---	-- T	--
SfMNPV	---	--G	---	--C	---	--G	--
SeMNPV	---	--A	---	--C	---	--A	--
OpMNPV	-- A	--A	---	--C	---	--G	--
HzMNPV	---	--G	---	--C	---	--A	--
AfMNPV	---	--G	--C	--C	---	-- T	--

Dash represents 100% similarity in relation to the primer sequence. Bases represent one of the two choices in a degenerate position. Bold bases represent a mismatch in relation to the primer sequence.

By analyzing the *polyhedrin* gene sequences in the Map program (from GCG package), we determined a number of restriction enzymes that produce distinct profiles for the different viral amplified DNAs. These enzymes and the size of fragments produced upon digestion of the viral PCR products are presented in Table 2.2. The enzymes HhaI, HincII and DdeI cut the majority of the viruses, yielding distinct profiles. Digestion with DdeI did not give good resolution for some PCR products because of closely migrating bands (SfMNPV and AfMNPV), the large number of bands (4 bands for SfMNPV, 5 bands for AfMNPV) and bands of low molecular weight (Data not shown). Also, DdeI

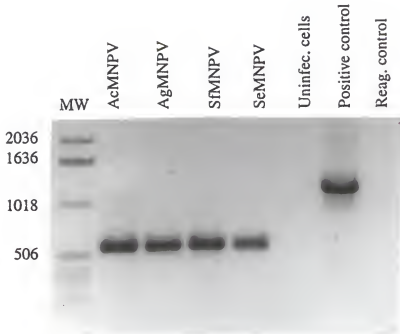


FIGURE 2.1: Specific PCR amplification of the *polyhedrin* gene coding region of four baculoviruses. Lane 1: molecular weight standard (1 kb ladder); Lane 2: AcMNPV; Lane 3: AgMNPV; Lane 4: SfMNPV; Lane 5: SeMNPV; Lane 6: uninfected SF9 cells; Lane 7: positive control (constructed plasmid containing the SfMNPV GP-41 gene); and Lane 8: reagents control (all the PCR reagents included, except template DNA). SF9 cells were infected with the respective viruses and incubated at 27°C for 48 hours.

Table 2.2: Expected fragments obtained after restriction digestion of PCR amplified viral DNAs.

Restriction Enzyme	VIRUSES									
	AcMNPV	BmMNPV	AgMNPV	SiMNPV	SeMNPV	OpMNPV	AMNPV	H2SNPV		
HhaI	516	516	203	313	-	204	516	354		
	59	59	189	203		203	59	221		
HincII			124	59		81				
			59			59				
						28				
	291	353	324	-	284	248	248	353		
	248	222	222		167	222	222	222		
DdeI			29		124	69	105			
						36				
	476	382		271	-	382	223	348		
	60	193		175		193	108	227		
	39			69			99			
				60			94			
							51			

did not cut two of the viruses (AgMNPV and SeMNPV). HhaI and HincII cut all the viral PCR products with the exception of SeMNPV and SfMNPV, respectively. HhaI yielded the same restriction profile for AcMNPV, BmMNPV, and AfMNPV, while HincII yielded the same restriction profile for the BmMNPV, and HzSNPV PCR products (Table 2.2). However, digestion with HhaI and HincII provided distinct profiles for the eight viral PCR products (Figures 2.2 and 2.3), and therefore, they can be used as a diagnostic tool, for the recognition and differentiation of these eight baculoviruses.

Discussion

In this study we have optimized the polymerase chain reaction for amplification of a target DNA sequence from multiple baculoviruses. Viral DNA obtained from infected larvae, infected insect cells, and commercial formulations were good sources of DNA for PCR amplification. The *polyhedrin* gene, which has about 80% sequence homology among NPVs (Rohrmann, 1986), proved to be a suitable gene for the development of a generic amplification technique. The *polyhedrin* gene also contains less conserved regions from which specific primers have been designed. Webb et al. (1991) analysed multiple clones of recombinant baculovirus and detected nonrecombinant virus contamination by using specific AcNPV primers that target the promoter region (-168 to -144) and the beginning of the coding region (+217 to +193). Burand et al. (1992) were able to amplify baculovirus DNA from OBs contaminating the surface of gypsy moth eggs by using specific AcNPV primers (forward: +6 to +25; reverse: +704 to +685) and specific *Lymantria dispar* NPV primers (forward: +8 to +27; reverse: +701 to +682). The authors observed that both primers amplify their homologous viral DNA, but the LdNPV

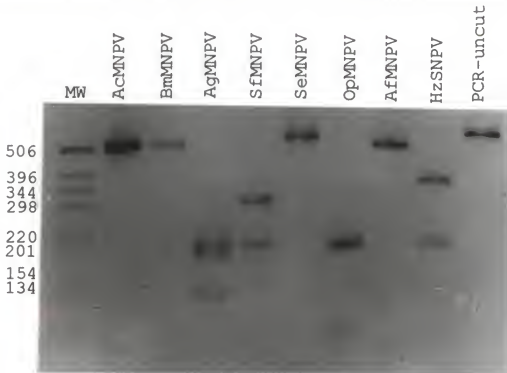


FIGURE 2.2: Restriction profile of eight baculovirus PCR products of the *polyhedrin* gene coding region digested with Hha I. The molecular weight standard used was the 1 kb ladder (lane 1).

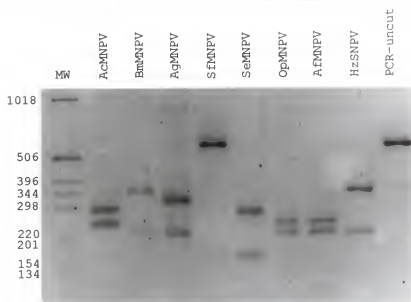


FIGURE 2.3: Restriction profile of eight baculovirus PCR products of the *polyhedrin* gene coding region digested with Hinc II. The molecular weight standard used was the 1 kb ladder (lane 1).

primers also amplify AcNPV sequences. In this study, our goal was to design primers which would amplify multiple baculoviruses, and therefore, we chose highly conserved sequences within the *polyhedrin* coding region (forward: +40 to +57; reverse: +614 to +597).

Our results suggested that the optimization of primer and magnesium concentrations were critical for baculovirus DNA PCR efficiency, whereas DNA-template concentrations produced positive amplifications within a broader concentration range. Ansari et al. (1992), detecting nucleic acids of the human immunodeficiency virus by PCR, also observed an increase in specificity and yield of the PCR product in response to an increase in Mg^{+2} concentration (3.0 mM) and decrease in primer concentration (25 ng/reaction). On the other hand, Wiedmann et al. (1993) reported that higher concentrations of magnesium (more than 1.5 mM) produced more non-specific amplification. Nonetheless, both authors emphasize the importance of a careful optimization of the PCR components, mainly magnesium and primers. Sensitivity of the PCR technique was not addressed in this study, but it will be the subject of future experiments. Sensitivity of PCR for baculovirus amplification has been reported by Burand et al. (1992), who were able to routinely detect the amplification of 5 copies of baculovirus genome in their studies. The choice of diagnostic restriction enzymes was critical because the objective was to distinguish the different viral species and to confirm the PCR results in a fast and economical way. Digestion of the different PCR products with only two restriction enzymes (HhaI and HincII) was sufficient to distinguish between them, providing a practical and economic diagnostic method.

In conclusion, we have developed a PCR procedure with primers capable of amplifying DNA from multiple baculoviruses. PCR amplification coupled with restriction enzyme analysis of the PCR products proved to be a specific and powerful technique that could be used in the future to study the environmental fate of wild type and/or genetically modified baculoviruses. The technique described in this chapter will be used to analyze field-collected samples containing baculoviruses, and this will be the subject of the next chapters.

CHAPTER 3
USE OF POLYMERASE CHAIN REACTION TO MONITOR AND TO STUDY
PERSISTENCE OF THE *ANTICARSLA GEMMATALIS* MULTIPLE-
NUCLEOPOLYHEDROVIRUS IN SOYBEAN FIELDS

Introduction

The velvetbean caterpillar, *Anticarsia gemmatalis* (Hübner), is an important defoliator in soybean crops in the southeastern United States and much of South America. The use of the *A. gemmatalis* multiple-nucleopolyhedrovirus (AgMNPV) to control the caterpillar in soybean fields in Brazil began in 1979, and represents one of the most successful examples of microbial control in the world. The program has steadily expanded with applications of the virus to over one million hectares of soybeans per growing season (Moscardi & Sosa-Gomez, 1993). Field trials conducted in Florida demonstrated that the Brazilian AgMNPV is efficacious against velvetbean caterpillar populations, although the levels of suppression were lower than those in Brazil (Funderburk et al., 1992).

The efficiency of baculoviruses as microbial control agents is dependent upon dispersion, transmission within the insect host population, and survival during host absence. Therefore, it is important to develop rapid and sensitive detection techniques for studying persistence and spread of baculoviruses.

Predators may be agents of baculovirus dispersion in the field, mainly because they excrete viable virus in their feces (Hostetter, 1971; Capinera & Barbosa, 1975; Beekman, 1980; Cooper, 1981; Abbas & Boucias, 1984; Young & Yearian, 1987; Vasconcelos et al., 1996). In soybeans, a number of predatory arthropods became contaminated with AgMNPV after the virus was released in the field (Boucias et al., 1987; Fuxa et al., 1993). *Nabis roseipennis* Reuter disseminated AgMNPV in a caged population of *A. gemmatilis* (Young & Yearian, 1992). In Florida, hemipterans such as bigeyed bugs (*Geocoris* spp.) and damsel bugs (*Reduviolus* spp. and *Nabis* spp.) are important predators in the soybean agroecosystem (Funderburk & Mack, 1987, 1989).

The detection and monitoring of intraspecific genetic variation in baculovirus field populations are also important. Wild type baculoviruses occur as a heterogeneous population in nature (Maruniak et al., 1984). One of the techniques routinely used to detect and characterize different viral isolates is plaque purification followed by restriction endonuclease analysis or Southern Blots (Miller & Dawes, 1978; Smith and Summers, 1978). Another alternative is to locate and characterize variable regions within the genome of interest and use them as molecular markers (Falk et al., 1995). Variable regions have been located in the AgMNPV genome by successive plaque-purification and restriction digestion of the most representative genomic variants (Maruniak et al., unpublished). One of these variable regions, located in the PstI-T fragment of the AgMNPV prototype (2D), has been analyzed and sequenced for two AgMNPV genomic variants, 2D and D7 (Garcia-Maruniak et al., 1996). DNA deletion or duplication events represented the differences between these two variants. AgMNPV-

2D contained 10 repeat elements of 127 to 128 bp, while, AgMNPV-D7 was 381 bp smaller, which represented a deletion of three complete 127 bp repeats. In addition, this AgMNPV repetitive region presented two 30 bp imperfect palindromes, showing similarities with the homologous regions (hrs) of some baculoviruses such as AcMNPV (Guarino et al., 1986), *Bombyx mori* MNPV (Majima et al., 1993), *Orgyia pseudotsugata* MNPV (Theilmann and Stewart, 1992), and *Lymantria dispar* MNPV (Pearson and Rohrmann, 1995). Previous reports have correlated the baculovirus homologous regions and the variable repetitive regions. For example, the homologous regions of *Choristoneura fumiferana* (CfMNPV) are interspersed in four locations throughout the genome and are formed by repeats of approximately 200 base pairs (Arif & Doerfler, 1984; Kuzio & Faulkner, 1984). Arif & Doerfler (1984) identified CfMNPV genomic variants, which had arisen from passages of the virus in the larval host, and presented different numbers of the 200 bp repeats. In the OpMNPV genome, a 66 bp element is tandemly repeated partially or completely 12 times (Theilmann & Stewart, 1992). The baculovirus homologous regions might function as transcriptional enhancers for early gene expression (Guarino & Summers, 1986a; Guarino et al., 1986; Nissen & Friesen, 1989; Carson et al., 1991; Guarino & Dong, 1991; Rodems & Friesen, 1993) or as origin of DNA replication (Pearson et al., 1992; Leisy & Rohrmann, 1993; Kool et al., 1993; Leisy et al., 1995). PCR amplification of the hr4 region of plaque-purified isolates obtained from AgMNPV wild-type preparations (1979, 1985, and 1994), showed that the isolates differ from each other by their number of 127 bp tandem repeats (Garcia-Maruniak, unpublished). These results indicate that the AgMNPV hr4 region is a

potential candidate area of the DNA for PCR amplification to detect different isolates of this virus.

The purpose of this study was to evaluate the PCR technique as a monitoring tool for determining persistence and spread of baculoviruses as well as for distinguishing isolates within the same species. We have chosen the AgMNPV as a model because of its potential for use as a biological control agent in the southeastern United States and its widespread use in Brazil.

Material and Methods

Virus

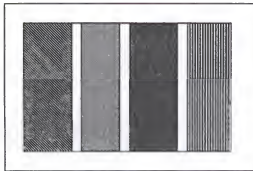
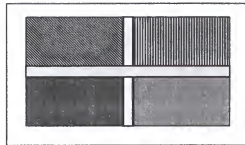
Two AgMNPV plaque-purified genomic variants, 2D and D7, were utilized in this study. AgMNPV-2D is considered the AgMNPV prototype because it represented the majority (40 %) of plaque-purified isolates obtained from an AgMNPV wild-type population from 1979 (Johnson & Maruniak, 1989). AgMNPV-D7, is a plaque-purified isolate obtained from a viral preparation that had been passaged 20 times in the alternate host *Diatrea saccharalis* (Pavan & Ribeiro, 1989).

Viral inocula to be applied in soybean plots was obtained by injecting 5 µl of extracellular virus of each variant in the hemocoel of fourth instars of *Anticarsia gemmatilis* (TCID₅₀ = 10⁶). Moribund larvae were frozen and polyhedra from the larval bodies were purified by maceration in homogenization buffer (1 % ascorbic acid, 2 % SDS, 0.01 M Tris pH 7.7, 0.001 M EDTA), followed by filtration through four layers of cheese cloth. The polyhedra was subsequently centrifuged twice at 4°C. The pellet was

resuspended and centrifuged in a sucrose gradient (40 %-63 %) to remove cellular debris. The band containing polyhedra was located in the lower third of the tube. The band was removed and the amount of polyhedra was estimated by counting in an hemacytometer chamber (Appendix A). These polyhedra preparations were applied separately and as a 1:1 mix in the field.

Field Conditions

During the 1995 growing season, genotypes AgMNPV-2D and AgMNPV-D7 were applied individually and as a mix in soybean plots at the North Florida Research and Education Center, University of Florida, Quincy. The dosage applied was 1.0×10^{11} polyhedra/ha as recommended for AgMNPV application (Moscardi, 1989). Half of the concentration of each virus, 5.0×10^{10} , was used for the viral mix application. The control was a fourth plot with no viral application. A schematic representation of the field design is shown in Figure 3.1. Soybean plots were 300 m^2 separated by a buffer zone of 30 m, and each treatment was replicated twice. The four outer soybean rows of each plot served as a border. The fungicide benomyl (DuPont, Inc., Wilmington, DE) was sprayed in the experimental area before viral application, in order to avoid *Nomuraea rileyi* (Farlow) Samson epizootics. The virus was applied on 08/30/95 and plots were sampled, by the ground cloth method (Funderburk et al., 1992), two days before and at 1, 10, 15, 30, and 45 days after virus application. Five random, 1-m row samples of *A. gemmatilis* larvae, and predatory damsel bugs (*Reduviolis* and *Nabis* spp.) and big-eyed bugs [*Geocoris punctipes* (Say)], were collected within each plot on each date. A total of

Block A**Block B****Legend**

AgMNPV-D7



AgMNPV-2D



Border and Buffer zone



AgMNPV 2D+D7



Control

Figure 3.1.: Schematic Representation of Field Design

50 larvae (if available), and as many predators as possible were collected per plot, put on ice, and taken to the laboratory to be frozen at -20°C .

Viral DNA Extraction from Individual Larvae and Individual Predators

Baculovirus DNA was extracted by maceration of individual larvae or predators in a homogenization buffer, and centrifuged at $3,000 \times g$ for 2 min to remove insect debris. The pellet was resuspended in $500 \mu\text{l}$ 1 M Tris-HCl pH8.0 and centrifuged at $10,333 \times g$ for 10 min. The pellet was resuspended in $500 \mu\text{l}$ 1 M Tris-HCl pH8.0, and it was treated in 1/3 of the total volume of an alkali solution ($0.3 \text{ M Na}_2\text{CO}_3$, 0.03 M EDTA pH 8.0, 0.51 M NaCl) to disrupt the polyhedra. The alkali-released virus was centrifuged at $10,333 \times g$ for 10 min and resuspended again in 1 M Tris-HCl pH 8.0 for neutralization purposes (Appendix E). These crude DNA preparations were diluted 100 x and used as templates for PCR amplification.

PCR Conditions

PCR amplifications for the polyhedrin region were performed in a total volume of 25 μl . The following reagents concentrations were used: 200 μM of each dNTP; 12.5 pmoles of each primer; 2.5 mM MgCl_2 ; and 0.5 Units of PrimeZyme (Biometra, Inc). The DNA template consisted of 1 μl aliquots of a baculovirus DNA preparation obtained from individual field-collected larvae or predators and diluted 100 times. The temperature program consisted of an initial denaturation step of $95^{\circ}\text{C}/1 \text{ min}$, followed by 35 cycles of $94^{\circ}\text{C}/1 \text{ min}$ (denaturation), $48^{\circ}\text{C}/1 \text{ min}$ and 10 s (annealing), $72^{\circ}\text{C}/1 \text{ min}$ and 30 s (extension). The final extension step lasted 15 minutes.

Polyhedrin PCR diagnosis was confirmed by digesting a direct aliquot of the PCR products with the restriction enzyme *HincII* using recommended conditions. Digested PCR products were visualized on a 3 % NuSieve GTG 3:1 gel (FMC, Inc.), stained with ethidium bromide in TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA pH 8.0).

The hr4 region was amplified using similar conditions as described for the polyhedrin PCR, with the exception of the concentration used for the $MgCl_2$ (1.5 mM) and primers (5 pmoles/primer). The temperature program for the hr4 amplification was also similar to the program described above, with the exception of the annealing step, which was held at 60°C. All PCR amplifications, were performed in a PTC-100 thermal cycler (MJ Research, Inc.). Polyhedrin and hr4 PCR products were visualized on a 0.75 % Seakem LE agarose gel (FMC, Inc.), stained with ethidium bromide in TAE buffer.

Primers

A set of degenerate primers for the AgMNPV and seven other baculovirus *polyhedrin* genes was designed using the Oligonucleotide Selection Program (OSP) (Hillier and Green, 1991). These primers (JM 33-JM34) were located within the *polyhedrin* gene coding region (forward: +40 to +57; reverse: +614 to +597) and their sequences were 5' TA(CT)GTGTA(CT)GA(CT)AACAA(GA)T3' and 5' TTGTA(GA)AAGTT(CT)TCCCA(AG)AT3' for the forward and reverse primers, respectively.

The hr4 primers were designed with aid of the Oligo 5.0 computer program (National Biosciences, Inc., Plymouth, MN). These primers (JM44-JM45) are located outside the hr4 region, and they are 100 % homologous for AgMNPV. Their DNA

sequences are 5' GCTACGCTTGTTCGGAAGT3' (forward) and 5' TGCAAATACAACGGGTCTCT3' (reverse).

Sensitivity of polyhedrin PCR

The sensitivity of the polyhedrin PCR was evaluated in the presence and absence of larval extracts. Aliquots of larval extracts (1 µl) obtained from larvae collected from soybean plots prior to baculovirus application and negative for polyhedrin PCR, were added to 1 µl of AgMNPV DNA purified from virions by standard procedures (Maruniak, 1986) and PCR-amplified for the *polyhedrin* gene. At the same time, the same concentrations of AgMNPV were amplified in the absence of larval extracts. PCR was performed in duplicates of the following concentrations: 1.0 ng, 0.5 ng, 0.05 ng, 5.0 pg, 0.5 pg, 50.0 fg, and 5.0 fg. The PCR conditions were identical to the described above.

Statistical Analysis

The data were transformed to square root of arcsine. A multiple analysis of variance (MANOVA) was performed, and the mean percentage of NPV detection in larval and predator samples was calculated using Tukey's test in the SAS software package (SAS Institute, 1989). Regression lines and partial correlation coefficients were also calculated by SAS, to determine possible significant associations between NPV detection in the larval host and in the predator population.

Sensitivity and specificity coefficients for intraspecific detection of AgMNPV were calculated on the SAS software package by using a Z test, which assumed approximate normality of the proportions. Sensitivity is defined as the proportion of times that a particular viral genotype was detected in a sample from a plot which had been sprayed with

that particular genotype, while specificity is defined as the proportion of times that a viral genotype was not detected in a plot not sprayed with that genotype (Agresti, 1990).

McNemar's test for matched proportions was used to compare efficiency of NPV detection by PCR amplification of two different regions of the AgMNPV genome (SAS institute, 1989).

Results

The population of *Anticarsia gemmatilis* larvae was above the economic threshold (27.7 larvae per sample) in block A by the time of viral application. The number of large caterpillars (larger than 1.5 cm) per sample was superior to 10, and baculovirus treatment is not usually recommended for such level of large larvae. Larval population in block B was much lower (9.8 larvae per sample) and it was represented by small and large larvae at approximately the same proportions. Soybean stand in block B was low in the control and AgMNPV 2D+D7 treatment plots, and the presence of weeds was high. These factors caused a discrepancy between the insect numbers in these two treatments and the insect numbers in treatments AgMNPV-2D and AgMNPV-D7. *A. gemmatilis* population in this area tended to be lower throughout the experiment.

PCR analysis of the *polyhedrin* gene coding region, which is capable of detecting multiple baculoviruses (Moraes & Maruniak, 1997), did not yield any positive amplification in the larvae and predator samples collected before virus applications. Therefore, the experimental area was considered free of detectable native baculoviruses. Maximum development of viral symptoms in *A. gemmatilis* larvae and maximum *A.*

gemmatalis larval mortality resulting from the viral applications in the soybean plots was observed 10 days after virus treatment (data not shown).

Sensitivity of Polyhedrin PCR

PCR for the polyhedrin region amplified as low as 50.0 fg of AgMNPV DNA in the absence of larval extracts and 0.5 pg in the presence of larval extracts, corresponding to 364 and 3.64×10^3 AgMNPV genome copies, respectively (Figure 3.2). The presence of larval extracts inhibited PCR by a 10 fold decrease in the amount of amplifiable AgMNPV DNA.

Detection and Environmental Fate of AgMNPV

The number of *A. gemmatalis* larvae collected to be PCR-amplified for the polyhedrin region were 217, 260, 299, and 224 for the control, AgMNPV-2D, AgMNPV-D7, and AgMNPV 2D+D7 plots, respectively. The size of the polyhedrin PCR product (575 bp) and the expected profiles of the PCR products when digested with the restriction enzyme HincII are shown in Figure 3.3. The enzyme HincII distinguishes AgMNPV from other baculovirus species, producing three fragments of 324, 222, and 29 bp. Digestion with HincII was performed in 20 % of the PCR reactions from field-collected larvae, and all digestions produced the expected profile for the AgMNPV polyhedrin amplification. Figure 3.3 also shows the PCR-product intensity and restriction enzyme analysis from field-collected larvae from one to 45 days post-application. At days 10 and 15, the products obtained were the most intense and corresponded to the peak of baculovirus disease and host mortality in the field. On the first day infection levels in the

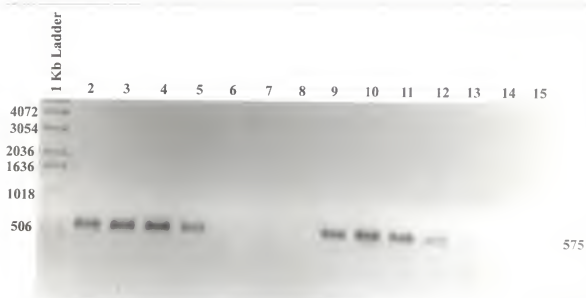


Figure 3.2: Sensitivity of polyhedrin PCR in the presence and in the absence of larval extracts. Lane 1: Molecular marker (1 kb Ladder); lanes 2-8: polyhedrin PCR products (575) amplified in the presence of larval extracts; lanes 9-15: polyhedrin PCR products amplified in the absence of larval extracts. Amounts of AgMNPV template DNA per PCR reaction were 1.0 ng (lanes 2 and 9), 0.5 ng (lanes 3 and 10), 0.05 ng (lanes 4 and 11), 5.0 pg (lanes 5 and 12), 0.5 pg (lanes 6 and 13), 50.0 fg (lanes 7 and 14), 5.0 fg (lanes 8 and 15). PCR products were visualized on 0.7% Seakem agarose gel stained with ethidium bromide in TAE buffer.

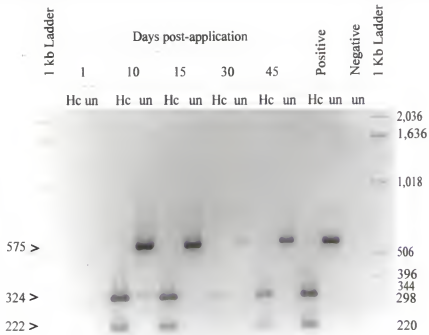


Figure 3.3: AgMNPV-2D polyhedrin PCR products uncut (un) and HincII profiles (Hc) for field-collected *A. gemmatalis* larvae. The molecular weight standard used was the 1 kb ladder (lanes 1 and 15). The sizes of the polyhedrin PCR product and HincII restriction fragments are indicated by arrows on the left side of the picture.

field were still low, and the resulting PCR bands were faintly seen in agarose gels. At 30 and 45 days, the PCR product intensity was still high. These results indicated that the virus cycled through the host generations, since the generation time for *A. gemmatalis* is around 24-28 days in a temperature range of 32-27°C, respectively (Leppä et al., 1977). Figure 3.4 shows the intensity of the polyhedrin PCR products for all the collected larvae throughout the experiment (1 to 45 days p.a.). The level of AgMNPV detection in its larval host per sampling date is shown in Figure 3.5. AgMNPV was detected in the host system from one to 45 days after baculovirus application in the field. No virus was detected in control plots one and 10 days after virus applications. However, baculovirus was detected in 5, 37, and 64% of the larval samples collected from control plots 15, 30 and 45 days after treatment, respectively (Figure 3.5). This indicates that the baculovirus dispersed at an average rate of approximately two to three meters per day, since there was a five-day interval between sampling dates, and the distance between plots was 30 meters. Tukey's test showed that the level of NPV detection did not differ significantly ($\alpha=0.05$) among treated plots, but detection was significantly lower in the control plots until 30 days after virus application.

A total of 201 predators (geocorids and nabids) were collected as follows: 31 (control), 48 (AgMNPV-2D), 55 (AgMNPV-D7), and 67 (AgMNPV 2D+D7). In predator samples, PCR analysis of the viral *polyhedrin* gene did not reveal virus at one day after baculovirus application, with the exception of the AgMNPV-D7 treatment. AgMNPV was first detected in the predator population at 10 days post-application and

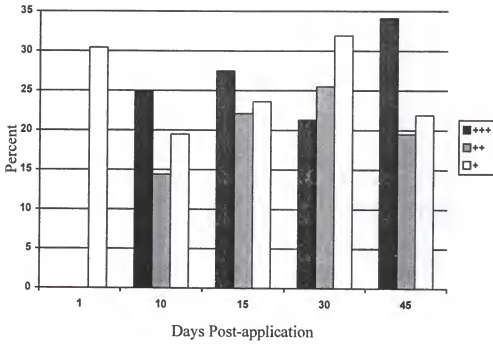


Figure 3.4: Intensity of polyhedrin PCR products obtained from *A. gemmatilis* larvae collected at five different sampling dates from AgMNPV-treated soybean plots.

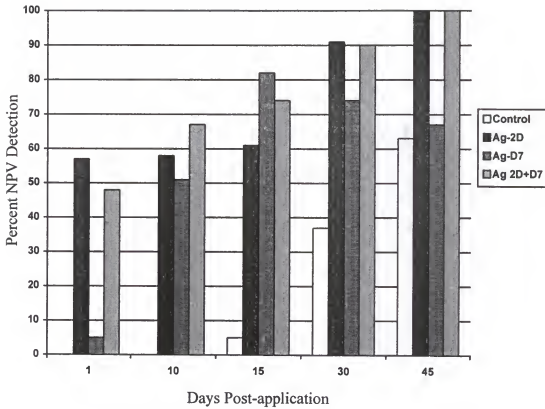


Figure 3.5: Percent PCR detection of the *Anticarsia gemmatilis* nuclear polyhedrosis virus (%) in its larval host, *A. gemmatilis*, monitoring the *polyhedrin* gene. Percentage values correspond to the total number of *A. gemmatilis* larvae collected per control or viral treatment in each sampling date.

continued until 45 days post-application (Figure 3.6). In control plots, the presence of AgMNPV in predator samples was first observed 10 days post-application, while the presence of AgMNPV in the larval host was observed only at 15 days post-application (Figure 3.5). This indicates that the virus was present at least five days earlier in the predator population. Percentage of AgMNPV detection in the predator population did not differ significantly among treated plots and control ($\alpha=0.05$). The coefficient of variation for this analysis was high (58.3), probably due to the low numbers and/or unequal distribution of predators in the field. There was no linear relationship between AgMNPV detection in larvae and in the predator population ($p=0.9754$). The partial correlation coefficient, which attempts to remove any date effect, did not show significant correlation between these two events.

Detection of AgMNPV genotypic variants in Soybean Fields

PCR amplification of the hr4 region appeared to be AgMNPV-specific, with our set of primers, since no amplification was observed when seven other baculovirus species, including AcMNPV, BmMNPV, SfMNPV, SeMNPV, OpMNPV, AfMNPV, and HzSNPV, were used as templates. However, PCR amplification of the polyhedrin region for the same viral species, produced products which were 575 bp in length (Figure 3.7). PCR targeting the hr4 region yielded products which were 1,726 bp and 1,345 bp for the AgMNPV-2D and AgMNPV-D7, respectively (Figure 3.7). AgMNPV was also detected from one to 45 days post-application in field-collected *A. gemmatilis* larvae, when the hr4 region was monitored by PCR amplification (Figure 3.8). However, a McNemar's test for matched proportions revealed that PCR of the polyhedrin region was

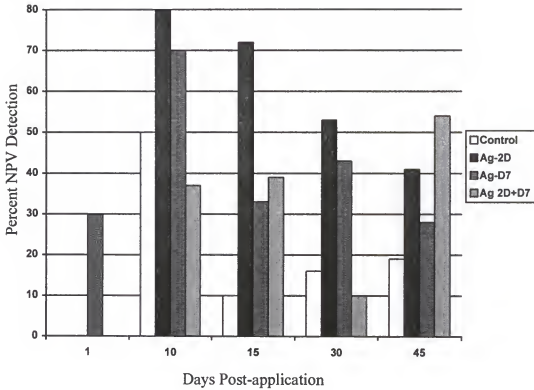


Figure 3.6: Percent PCR detection of the *Anticarsia gemmatilis* nuclear polyhedrosis virus (%) in *Nabis* spp. and *Geocoris* spp. Percentage values correspond to the total number of predators collected per control or viral treatment in each sampling date. Target genomic region was the *polyhedrin* gene.

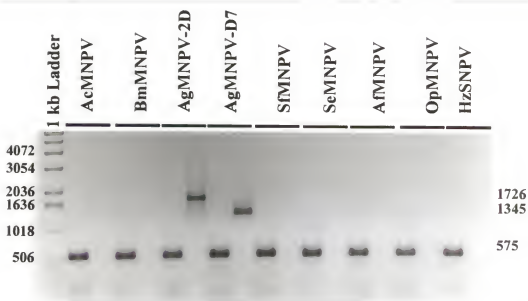


Figure 3.7: PCR amplification of the highly variable region 4 for the AgMNPV genomic variants 2D and D7 and seven other baculovirus species. The molecular weight standard used was the 1 kb ladder. The size of the polyhedrin-PCR product is 575 bp, and the size of the products for the hr4 region is 1726 bp and 1345 bp for the AgMNPV-2D and AgMNPV-D7, respectively. PCR products were visualized on 0.7% Seakem agarose gel stained with ethidium bromide in TAE buffer.

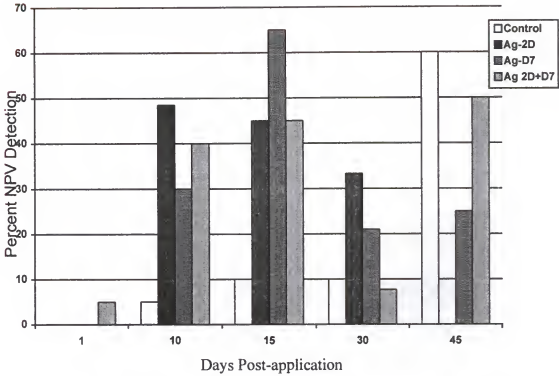


Figure 3.8: Percent PCR detection of the *Anticarsia gemmatalis* nuclear polyhedrosis virus (%) in its larval host, *A. gemmatalis*, monitoring the hr4 region. Percentage values correspond to the total number of *A. gemmatalis* larvae collected per control or viral treatment in each sampling date.

significantly more sensitive ($\alpha=0.05$) in detecting AgMNPV in its larval host than PCR for the hr4 region (data not shown).

Nonetheless, the objective to distinguish the two AgMNPV genomic variants applied in soybean plots was accomplished by PCR amplification of the hr4 region. Figure 3.9 shows the detection of AgMNPV-2D and AgMNPV-D7. In plots where AgMNPV-2D was originally applied, 96.5 % of the virus detected was the AgMNPV-2D genotype. Comparably, AgMNPV-D7 represented 82 % of the virus detected in the plots receiving only this genotype. The small percentage of detection of genotypes not sprayed in the plots can be attributed to virus movement among the plots. PCR analysis of the hr4 region distinguished successfully the two AgMNPV genotypes when they were applied into separate soybean plots. However, when both genotypes were applied as a 1:1 mix, we observed a differential detection. From the total virus detected in the AgMNPV 2D+D7 plot, 70 % corresponded to the AgMNPV-D7 genotype, and only 30 % corresponded to AgMNPV-2D.

Sensitivity and specificity coefficients for AgMNPV PCR detection, when the hr4 region was targeted are presented in Table 3.1. The hr4 PCR was highly specific for detection of AgMNPV-2D and AgMNPV-D7, presenting coefficients larger than 0.9 (coefficients vary from 0 to 1). On the other hand, sensitivity coefficients were low, especially in detecting AgMNPV-2D when AgMNPV-D7 was also present.

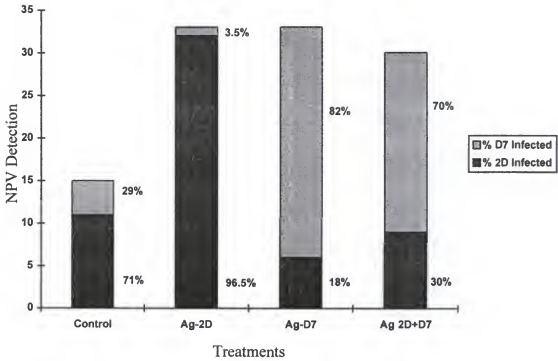


Figure 3.9: Detection of AgMNPV genotypes by PCR targeting the hr4 region. Sampling dates were combined for each treatment over a 45 day period, resulting in an overall NPV detection per treatment.

Table 3.1: Specificity and sensitivity of AgMNPV detection at the intraspecific level .

Treatments	Specificity		Sensitivity	
	AgNPV2D	AgNPVD7	AgNPV2D	AgNPVD7
Control	0.89	0.96	-	-
AgNPV-2D	-	0.99	0.32	-
AgNPV-D7	0.94	-	-	0.27
2D+D7	-	-	0.08	0.20

Coefficients were calculated by using a Z test, assuming approximate normality of the proportions. Data were computed using SAS software package.

Discussion

In this study we developed a rapid DNA extraction procedure to extract AgMNPV DNA from individual larva or predator which was suitable for PCR analysis. PCR for the *polyhedrin* gene amplified as low as 3.64×10^3 AgMNPV genome copies added to larval extracts. The larval extracts inhibited PCR to some extent, since a lower number of genome copies (364 genome copies) was PCR-amplifiable in the absence of larval extracts. The inhibitory effects of insect extracts in baculovirus detection has been reported by authors using ELISA as a detection technique (Longworth & Carey, 1980; Quant et al., 1984). The detection technique presented in this study was more sensitive

than dot blot hybridization assays, in which the lowest detection levels were 1.0 to 0.1 ng of NPV DNA (Ward et al., 1987; Keating et al., 1989; Kaupp & Ebling, 1993; Kukan & Myers, 1995). However, our PCR technique was less sensitive than a PCR protocol developed by Burand et al. (1992), which detected 5 genome copies from viral polyhedra contaminating the surface of gypsy moth eggs during 40 cycles of PCR amplification. Our detection limit was 364 AgMNPV genome copies for 35 cycles of PCR amplification. It is possible that the sensitivity of our system could have been increased by running PCR for more cycles (40-45 cycles). The PCR sensitivity could have been certainly increased by a second round of PCR amplification with the same primer pair or with internal primers (nested PCR), or by hybridizing the PCR products with a specific radiolabeled probe. However, these options would increase labor and time spent with the detection protocol, being contrary to one of the objectives of this study, which was to develop a rapid detection system for baculoviruses. In addition, a second round of PCR amplification increases the probability of contamination, and the use of radiolabeled probes increases safety concerns.

One application of AgMNPV in the treated plots was sufficient to maintain NPV infection in the *A. gemmatilis* population until soybean senescence. Similar results have been reported by Fuxa et al. (1993). In Brazil, where this NPV is widely used, one application per season is usually enough to effectively control velvetbean caterpillar populations (Moscardi, 1989). Maintenance of AgMNPV infection in the field is mainly a result of three factors: 1) horizontal transmission; 2) virus movement; and 3) persistence. In the present work, we studied these factors by PCR amplification of the

viral *polyhedrin* gene. The intensity of the PCR products in the laboratory allowed us to make inferences about the level of NPV infection in the field. It was clear that the virus cycled through host generations, since PCR products of high yield were observed from 10 to 45 days post-application.

In this study, our primary goals did not include the elucidation of the rate of spread nor the maximum distance of spread of AgMNPV. However, the potential of the PCR technique for dispersal studies was demonstrated by detection of AgMNPV movement from the treated plots into the control (PCR for the polyhedrin region), as well as among treated plots (PCR of the hr4 region). The virus was expected to move into the control plots because the distance between plots was approximately 30 m. Previous reports have shown that AgMNPV spreads at least 69 m (Richter & Fuxa, 1984), and 44-58 m (Fuxa & Richter, 1994) during one soybean growing season. The present study indicated that AgMNPV spread at an average rate of two to three m per day. This is based on the fact that the distance between plots was about 30 m, and the virus was first detected in control plots at 10 and 15 days post-application in the predator and host populations, respectively. The same virus spread at a rate of approximately one m per day in soybean fields in Louisiana (Fuxa & Richter, 1994).

The predators collected in this study were hemipterans from the families Nabidae and Lygaeidae, which are among the most abundant in the soybean ecosystem (Boucias et al., 1987; Funderburk & Mack, 1987, 1989; Fuxa et al., 1993). In this study, AgMNPV was detected in predators in control plots at least five days before detection in the host population. This indicates that predators were probably involved in the transport and

establishment of NPV infection in *A. gemmatilis* larvae in the control plots. These results agree with many reports which implicate predators as agents of baculovirus dispersion in the field. Many predators excrete viable virus in their feces, including the carabid beetles, *Calosoma sycophanta* L. (Capinera & Barbosa, 1975), *Harpalus rufipes* De Geer, *Pterostichus melanarius* Illiger and *Agonum dorsale* Pont. (Vasconcelos et al., 1996); sarcophagid flies (Hostetter, 1971); and the hemipterans *Oechalia schellenbergii* (Guerin-Meneville) (Cooper, 1981); *Nabis tasmanicus* (Het.) (Beekman, 1980), *Podisus maculiventris* (Say) (Abbas & Boucias, 1984); and *Nabis roseipennis* Reuter (Young & Yearian, 1987). *N. roseipennis* was found to disseminate AgMNPV in a caged population of *A. gemmatilis* (Young & Yearian, 1992). In another study, soil bioassays demonstrated that carabid beetles continuously passed infective virus to the soil for at least 15 days after feeding on infected *Mamestra brassicae* larvae (Vasconcelos et al., 1996). The acceptance of NPV-infected larvae as a food source is variable. Predators either show no discrimination between healthy and diseased larvae (Abbas & Boucias, 1984; Vasconcelos et al., 1996), or strongly prefer infected larvae as prey (Young & Yearian, 1987, 1989). In this study, the highest percentage of AgMNPV detection in the predator population occurred from 10 to 45 days post-application, indicating that predators acquired virus by feeding upon infected-*A. gemmatilis* larvae. If predators had become contaminated with AgMNPV due to its application in the field, it would be expected that the highest levels of contamination would have occurred at day one after virus application, and it would have decreased quickly thereafter. Fuxa et al. (1993) have reported similar results for predators collected in soybean fields in Louisiana.

We successfully used PCR of the hr4 region to distinguish AgMNPV genotypes under field conditions, indicating that this region in the AgMNPV genome is a good candidate to detect genetic intraspecific variation in populations of this virus. However, differential detection was observed when AgMNPV-2D and D7 were applied together. There are at least two possible explanations for these results: 1) differences in PCR amplification efficiency of the two genotypes; and 2) differential replication within the insect host. PCR amplifies small products more efficiently (McCulloch et al., 1995; Lee et al., 1996), which reinforces our first hypothesis. The specificity and sensitivity data strongly agreed with the PCR results. Low sensitivity coefficients were observed, especially, when both genotypes had been applied together. Certainly, other variable regions should be identified and characterized for the AgMNPV and other baculovirus species to be used as molecular markers, and to identify a larger range of variability in field populations.

Several techniques have been used to detect baculoviruses, including microscopy, bioassays, serological methods and DNA hybridization. Microscopic analysis and bioassays are time consuming, and the earliest baculovirus detection is about two to four days post-infection (Longworth & Carey, 1980; Entwistle & Evans, 1985; Evans, 1986). Virus identification in bioassays is only tentative and is based on susceptibility of a particular host species. Serological methods and DNA hybridization present limitations due to cross reaction problems (Smith & Summers, 1981; McCarthy & Henchal, 1983), and use of radioactive materials (Ward et al., 1987; Keating et al., 1989; Kukan & Myers, 1995), respectively. We developed a non-radioactive detection system, based on the polymerase chain reaction, which detected AgMNPV in its host at an early phase of

disease development (1 day), and for an extended period of time (45 days). In previous work, we identified eight baculovirus species using degenerate PCR primers, which targeted the coding region of the *polyhedrin* gene (Moraes & Maruniak, 1997). In the present study, PCR detection was expanded to the intraspecific level by PCR amplification of the *hr4* region. The ability to detect baculoviruses in field populations, at inter- and intraspecific levels, could have important applications in risk assessment and in the quality control of programs utilizing both wild type viruses and recombinant baculoviruses. Good quality control is an essential element in the development and maintenance of baculovirus efficacy in current and future insect pest management programs.

CHAPTER 4

ANTICARSIA GEMMATALIS BACULOVIRUS DETECTION FROM SOIL SAMPLES

Introduction

The soil is a long term reservoir for baculoviruses in the environment, regardless of the life style of the host, type of plant on which it feeds, or mode of action of the virus (Evans & Harrap, 1982; Entwistle & Evans, 1985). Many studies have reported persistence of baculoviruses in the soil for periods up to five years (David & Gardiner, 1967; Jaques, 1967; Mohamed et al., 1982). Therefore, the ability to detect and to quantify baculovirus polyhedra or DNA in the soil should contribute to a better understanding of baculovirus epizootics. The detection and quantification of baculovirus DNA, in particular, would be useful in risk assessment studies.

The detection of baculovirus polyhedra from soil varies in sensitivity according to soil type (Fuxa et al., 1985; Fuxa & Richter, 1993), and soil pH (Hukuhara & Wada, 1972). Polyhedra are absorbed by soil particles mainly by Coulomb forces; that is, negatively charged polyhedra are retained on the positively charged sites on the soil particles (Hukuhara & Wada, 1972). Fuxa et al. (1985) were able to detect 4×10^4 polyhedra per gram of soil in sandy soils, however, the detection limit was 10 polyhedra/g when the soil contained a high content of silt or clay. In another study, Fuxa & Richter (1993) detected 16 AgMNPV polyhedra/g in a soil containing 2.4% sand,

76.0% silt, and 21.6% clay, and 318 polyhedra/g in a soil composed of 25.0% sand, 54.0% silt, and 20.9% clay. Wood et al. (1994) detected as little as 7 polyhedra of AcMNPV per gram of soil. However, there was no mention of soil type in this work.

Different reagents have been used to extract baculovirus polyhedra from soil, however, the efficiency of polyhedra recovery has always been low. Hukuhara & Wada (1972) found that the most effective reagents to desorb polyhedra of a cytoplasmic polyhedrosis virus from soil were (in decreasing order): sodium pyrophosphate, Na-EDTA, and sodium oxalate. The percent recovery of CPV polyhedra from soil using 50 mM sodium pyrophosphate was 7% (Hukuhara, 1975). More recently, AcMNPV polyhedra was extracted from soil samples using a 0.1% SDS solution, with an overall extraction efficiency of 24% (Wood et al., 1994).

There are no reports on the direct detection of baculovirus DNA from soil. Nonetheless, DNA from several bacteria (Jacobsen & Rasmussen, 1992; Picard et al., 1992; Jacobsen, 1995; Volossiouk et al., 1995; Berthelet et al., 1996), and viruses such as enteroviruses (Bitton et al., 1979; Farrah et al., 1981; Straub et al., 1994), were directly extracted from soil and sediments and amplified by the polymerase chain reaction. The main challenge regarding extraction of DNA from organisms living in the soil is the presence of humic acids and phenolic compounds that are co-extracted with the DNA and are inhibitory to enzymes used in DNA manipulation, such as restriction endonucleases and DNA polymerases (Tsai & Olson, 1992; Tebbe & Vahjen, 1993). There are many reports on DNA extraction from soil microorganisms. Bitton et al. (1979) tested nine different eluents, and found that 0.5 % isoelectric casein and 0.5 % non-fat dry milk were

the most efficient in desorbing viruses from soil. Farrah et al. (1981) reported that solutions of 4 M Urea buffered at pH 9.0 with 0.05 M lysine were able to elute 70 % of the poliovirus adsorbed to sludge. Straub et al. (1994) used size-exclusion chromatography and ion-exchange chromatography to extract enterovirus DNA from sludge-amended soil and to remove compounds inhibitory to PCR. Picard et al. (1992) and Volossiuk et al. (1995) reported on the use of polyvinylpyrrolidone (PVPP) and skim milk powder to extract bacterial DNA, and to remove humic acids and other phenolic impurities from soil. Sensitive detection of bacterial DNA in the soil, and complete removal of humic acids was accomplished using a magnetic capture-hybridization and PCR amplification assay (Jacobsen, 1995). This method uses streptavidin-coated magnetic beads conjugated to a biotinylated probe which is specific to the target DNA. After conjugation of the internal probe to the magnetic beads, the coated beads are mixed with the DNA solution to hybridize with target DNA sequences. By application of a magnetic field, the beads containing the target DNA are then removed from nontarget DNA and interfering compounds.

Competitive PCR (cPCR) for the quantitation of DNA or RNA has been widely used (Harlow & Stewart, 1993; Leser et al., 1995; Schneeberger et al., 1995; Wieland et al., 1996). In this method, an internal standard or competitor is co-amplified with the target DNA or RNA using a common set of primers (Harlow & Stewart, 1993; McCulloch et al., 1995; Zimmermann & Mannhalter, 1996). If the efficiency of amplification of the two species is the same, the ratio of products following PCR reflects the initial amounts present and it enables quantitation (McCulloch et al., 1995). For DNA

quantitation, the competitor is usually a double stranded DNA molecule identical to the target sequence with either a small deletion, insertion, or new restriction site, and it presents the same primer binding sites as the target sequence (Harlow & Stewart, 1993).

The objectives of this study were to develop a methodology to extract baculovirus DNA from soil for further PCR amplification; to use the developed methodology to detect baculovirus DNA from field-soil samples collected over one year period after virus application; and to quantify baculovirus DNA from field-collected soil samples, using a competitive PCR procedure. The results of this work can be used to help elucidate the environmental fate of baculoviruses used as microbial control agents.

Material and Methods

Polyhedral Extraction and Viral DNA Purification

A series of experiments were performed to optimize extraction of baculovirus DNA from soil samples for subsequent PCR amplification. Different treatments consisted of 0.25 g of autoclaved soil inoculated with 50 μ l of AgMNPV polyhedra suspension. The viral concentrations tested were 1.0×10^7 , 1.0×10^5 , 1.0×10^4 , 1.0×10^3 , 1.0×10^2 , 1×10^1 , and 1 polyhedra per 0.25 g of soil. The latter concentration of virus was only tested for the magnetic capture-hybridization (MCH) experiments. Two different methodologies were tested, and their efficiency to extract baculovirus DNA was compared.

Phenol-ether extraction

This method consisted of extraction of humic acids by incubating soil samples in a solution of 1 M sodium pyrophosphate ($\text{Na}_4\text{P}_2\text{O}_7$) for four hours in a shaker at room

temperature. Subsequently, AgMNPV polyhedra were disrupted by two hours incubation with a dilute alkaline solution plus 0.2 M NaOH. Sodium hydroxide is also known to extract humic acids from soil. The alkali released virus was incubated with Proteinase K (5 mg/ml) overnight at 37°C, or for two hours at 65°C in order to degrade the viral envelopes and capsids. The virus DNA was then purified by successive phenol-ether extractions (Appendix F).

Magnetic capture-hybridization (MCH)

This method was modified from Jacobsen (1995, 1996). It consisted of streptavidin-coated magnetic beads (Dynal, Inc., Lake Success, NY) conjugated to a biotinylated probe (DuPont, Inc., Wilmington, DE) specific for the AgMNPV polyhedrin region (+240 to +343), to capture AgMNPV DNA by hybridization from soil samples. The biotinylated probe contained a biotin molecule on a five-carbon atom spacer arm incorporated at the 5' end of the oligonucleotide. The oligonucleotide was 103 bp long, and it was purified by high pressure liquid chromatography (HPLC). The magnetic beads were conjugated to the biotinylated probe by 1 hour incubation at room temperature in a Mini Hybridization Oven OV3 (Biometra, Inc., Tampa, FL). The conjugated beads were incubated for 15 min in the hybridization oven at room temperature in 400 µl of 0.125 M NaOH, 0.1 M NaCl. After this incubation, the conjugated beads were washed three times with 400 µl TE, 1 M NaCl to remove any NaOH and residual complementary DNA. The beads were then resuspended in sterile distilled (SD) H₂O and used for hybridization (Appendix G). Soil containing different concentrations of AgMNPV polyhedra (as described above) was incubated in a shaker for one hour at room

temperature with a combination of TE buffer, dilute alkaline solution, and 0.2 M NaOH in order to disrupt the polyhedra. The soil samples were centrifuged at $3,000 \times g$ for 5 min. The supernatant was centrifuged again at $10,330 \times g$ for 20 min at 4°C . The pellet was resuspended in $1 \times$ PBS buffer and boiled for 10 min to disrupt the virus envelope and capsids. After boiling, the samples were centrifuged at $10,330 \times g$ for 10 min at 4°C . The supernatants were saved and used for hybridization to the biotinylated probe (Appendix H). Hybridization took place in a hybridization oven at 62°C for 1.5-2 hours, according to the calculated number of hours to reach $2 \times \text{Cot}1/2$ (Sambrook et al., 1989). After hybridization, the beads were washed with $400 \mu\text{l}$ of SD H_2O and resuspended in a final volume of $50 \mu\text{l}$ SD H_2O . For PCR amplification, $25 \mu\text{l}$ of resuspended beads were added to $25 \mu\text{l}$ of PCR master mix.

PCR Conditions

PCR conditions were identical to those described in chapters 2 and 3, when the target DNA was obtained by the phenol-ether procedure. DNA-template was diluted 10 or 100 times. When the template DNA was obtained through the MCH procedure, PCR was performed in a total volume of $50 \mu\text{l}$, containing $25 \mu\text{l}$ of resuspended beads and $25 \mu\text{l}$ of PCR master mix (Appendix I). Each reaction tube was covered with $50 \mu\text{l}$ of mineral oil to prevent evaporation. The temperature program was the same described in chapters 2 and 3, but it was run for 40 cycles instead of 35.

The primer sequences (JM33 and JM34) were derived from conserved sequences in the 5' end of the coding region of the *polyhedrin* gene (Moraes & Maruniak, 1997). This set of primers is 100% homologous to AgMNPV, and their sequences have been

described in previous chapters. The amplification products were analyzed by 0.7% Seakem LE agarose (FMC, Inc.) gel electrophoresis in TAE buffer stained with ethidium bromide.

Collection of Field Soil Samples

The field experiment was detailed in the Material and Methods section of Chapter 3. The soil was a Dothan loamy fine sand, and according to the USDA textural triangle classification, it contains 70-85% sand, 10-20% clay, and 0-30% silt. Soil samples from the top 15 cm were collected with the aid of a core sampler within five sampling sites in each plot. Each soil sample weighed 69 g on average. Control and AgMNPV-2D treatments were sampled at 1, 15, 45, 75, 180, and 330 days after virus application. The soil samples were collected in individual plastic bags, put on ice, and taken to the laboratory to be frozen at -20°C. In the laboratory, each soil sample was homogenized manually, and a subsample of 0.5 g was used for baculovirus DNA extraction by the MCH procedure. Aliquots of the extracted DNA were used in PCR amplification experiments for detection of AgMNPV. PCR amplification targeted the coding region of the *polyhedrin* gene as described before.

Competitive PCR (cPCR)

To quantitate AgMNPV DNA from soil samples a competitive PCR (cPCR) approach was used. In this strategy, the wild-type AgMNPV *polyhedrin* gene was considered the target DNA, and a competitor DNA containing an internal deletion of 65 bp in relation to the target DNA was constructed. Since the competitor DNA has the same sequence as the wild type target DNA, except for the 65 bp deletion, it should

maintain the same primer binding sites as the wild-type, and therefore, render amplification by the same primers used for PCR amplification of the wild-type *polyhedrin* gene. The size of the expected PCR products is 575 and 510 bp for the AgMNPV target DNA and competitor DNA, respectively.

The first step in the quantitation protocol is achieved by co-amplifying unknown amounts of target DNA with fixed amounts of competitor DNA. If the efficiency of the PCR amplification of the two DNA species is the same, the ratio of products following PCR reflects the initial amounts of target DNA. The second step is to separate and to visualize the target and competitor PCR products by gel electrophoresis and UV transillumination. The third step is to blot the resulting PCR bands in the gel to a nitrocellulose membrane and hybridize it to a ^{32}P radiolabeled probe specific for the AgMNPV *polyhedrin* gene. In the fourth step, the hybridized membrane is exposed to a PhosphorImager cassette, scanned in a PhosphorImager, and the separated bands corresponding to amplified target and competitor DNAs are quantified by volume integration (ImageQuant software v 3.0; Molecular Dynamics, Inc.). Details on the construction of the competitor DNA and AgMNPV DNA quantitation by cPCR of the *polyhedrin* gene are given below.

Construction of Competitor DNA

Pretreatment of PCR product

The 575 bp *polyhedrin* PCR product was treated with Proteinase K (5 mg/ml) in 100 mM Tris-HCl, 50 mM EDTA, 1% SDS to remove the PrimeZyme DNA polymerase bound to the DNA (Crowe et al., 1991). The Proteinase K digestion was carried out at

60-68°C for one hour. The Proteinase K treated PCR product was cleaned using the QIAquick PCR Purification Kit (QIAGEN, Inc., Chatsworth, CA).

Restriction enzyme digest

The polyhedrin PCR product was digested with the restriction enzyme AluI (New England Biolabs, Inc., Beverly MA). For the digest, 15 µl of amplified DNA (300-500 ng), 0.5 µl of AluI enzyme (8 U/ µl), and 2 µl of the appropriate 10x restriction buffer (manufacturer's instructions) were mixed and incubated overnight at 37°C. After digestion, the enzyme was heat-inactivated at 65°C for 10 min. Digestion of the PCR product with this enzyme produced fragments of 297, 213, and 65 bp which were separated on 3% NuSieve GTG: Seaplaque agarose gel (FMC, Inc.).

Ligation of fragments

Fragments of 297 and 213 were excised from the gel, purified using the QIAquick Gel Extraction Kit (QIAGEN, Inc.), and then ligated using T4 DNA Ligase (Promega, Inc., Madison, WI). The ligation reaction was based on Sambrook et al. (1989), and it was carried out in 31 µl, including 20 µl of fragments 297 and 213 bp (approximately 100 ng of each DNA fragment), 1 µl T4 DNA ligase (3 U/µl), 3 µl 10x reaction buffer, and 7 µl 40% Polyethylene Glycol (PEG₈₀₀₀) for 16 hours at 15°C. The efficiency of blunt-end ligations is improved by adding PEG₈₀₀₀ in a final concentration of 15% (Sambrook et al., 1989). After ligation, the enzyme was inactivated at 65°C for 10 min, and PEG₈₀₀₀ was removed by sequential extractions with phenol, chloroform, and ether. The expected ligation product should be 510 bp long, due to a 65 bp deletion in comparison to the wild-

type polyhedrin PCR product. The ligated products were amplified using PCR primers for the polyhedrin gene (JM33 and JM34) to confirm ligation.

Cloning

The PCR-amplified 510 bp fragment was cloned into a pGEM-T Vector (Promega, Inc.) following the manufacturer's instructions. Before cloning, the 510 bp PCR product was pretreated with Proteinase K and cleaned using the QIAquick PCR Purification Kit, as described above. A 3:1 molar ratio of vector:PCR product DNA was used according to the manufacturer's instructions. The ligation system contained 50 ng pGEM-T Vector (1 µl), 25 ng of PCR product (2.5 µl), 1 µl T4 DNA ligase (3 U/ µl), and 1 µl 10x ligase buffer. Sterile-distilled H₂O (4.5 µl) was added to complete the volume to 10 µl. Ligation took place at 15°C for 16 hours. After ligation, the enzyme was heat-inactivated as described earlier. Subsequently, the ligated plasmid was diluted 5x and sterilized by adding 300 µl ether which was evaporated inside a hood. The plasmid containing the 510 bp insert was named pGEMPOL₅₁₀.

Transformation of *E. coli* DH5α competent cells

The transformation procedures were based on the recommendations by the competent cell's manufacturer. A 50 µl aliquot of *E. coli* DH5α competent cells (Life Technologies, Inc., Gaithersburg, MD) was thawed on ice, and 5 µl of 5x diluted DNA ligation system were gently mixed with the cells. The cells were sequentially incubated on ice for 30 min, heat shocked at 37°C for 30 sec, and cooled on ice for 2 min. Subsequently, 0.95 ml of room-temperature superoptimal catabolite (S.O.C) media was added. The cells were incubated in a shaker at 225 rpm at 37°C for 1 hour for antibiotic

expression. Aliquots of 100 and 200 μ l of transformed cells were plated on Petri dishes containing Luria-Bertani (LB) agar media, 100 μ g/ml ampicillin, and 20 μ g/ml 5-Bromo-4-chloro-3-indolyl-b-D-galactoside (x-gal), and these were incubated overnight at 37°C (Maniatis et al., 1989). Positive, ampicillin-resistant clones containing the 510 bp DNA insert were selected by their white color.

Glycerol stock from positive clones

According to Maniatis et al. (1989), white colonies were picked from the LB plate and grown in 3 ml LB liquid media supplemented with 100 μ l ampicillin (100 μ g/ml) for 16 hours at 37°C and 225 rpm. Subsequently, 850 μ l of the cell suspension was added to 150 μ l of sterile glycerol to make a 15% glycerol stock for further use. These procedures were performed inside a hood. The glycerol stock was stored at -70°C.

Plasmid DNA purification and confirmation of positive clones

The remaining cells containing the pGEMPOL₅₁₀ plasmid DNA were purified using the QIAprep Spin Plasmid Miniprep Kit (QIAGEN, Inc.) following the manufacturer's instructions. Aliquots of 2 μ l of each sample of purified plasmid DNA were run on 0.7% Seakem LE agarose gel electrophoresis to estimate DNA concentration. Plasmid DNA was digested with the enzymes PstI and SphI (New England Biolab, Inc.) to confirm insertion of the 510 bp deletion mutant. These two enzymes should excise the insert and produce two DNA fragments of 3.0 kbp and 510 bp, corresponding to the linearized pGEM-T Vector and to the insert fragment, respectively. After digestion for two hours at 37°C, the enzymes were heat-inactivated at 65°C for 10 min. Digested products were visualized on 3% NuSieve GTG 3:1 (FMC, Inc.) gel electrophoresis in

TAE buffer stained with ethidium bromide. The presence of the expected insert was further confirmed by PCR amplification of the *polyhedrin* gene using primers JM33 and JM34.

Test for Heteroduplex Formation

A procedure based on McCulloch et al. (1995) was performed to determine if the AgMNPV polyhedrin target and the competitor PCR products would form heteroduplexes during PCR amplification. A mixture of the 510 bp competitor and the 575 bp target PCR products was prepared at equal concentrations that could be readily visualized by agarose gel (120 ng of each PCR product). Half of this mixture was heated to 95°C for 5 min on a hot plate, and cooled to ambient temperature. The other half was not heated. The test was conducted in duplicate. The treated and non-treated mixtures were electrophoresed on 2 % Seakem LE agarose gel, and the presence or absence of extra DNA bands was observed. Two bands of equal intensity were expected if no heteroduplexes were formed.

Test for Differences in Amplification Efficiencies

A procedure according to McCulloch et al. (1995) was performed to determine whether the PCR amplification efficiencies for the AgMNPV polyhedrin target and competitor DNA were equal. Triplicates of a mixture of equal concentrations of competitor and target PCR were diluted 1 in 10⁶, and 1 µl aliquots were reamplified. The PCR products for both DNA species were separated by gel electrophoresis and visualized by UV light. The bands corresponding to target and competitor PCR products were blotted to a nitrocellulose membrane and hybridized to a ³²P radioactive polyhedrin

probe. The membrane was exposed to a PhosphorImager cassette and the resulting images, corresponding to amplified products for the target and competitor DNA, were scanned in the PhosphorImager. The PhosphorImager quantified the intensity of the PCR bands by volume integration (Molecular Dynamics, Inc., Sunnyvale, CA). The resulting values were entered in the Microsoft Excel v 5.0c spreadsheet (Microsoft, Inc., Redmond, WA) to calculate the ratios of competitor to target PCR products. A change in the ratio after amplification provided a direct measure of amplification efficiency in the absence of heteroduplex formation.

Standard Curve Construction

A series of tubes each containing 1.0 pg of the 510 bp competitor DNA (10^5 plasmid copies) and a series of dilutions of AgMNPV polyhedrin target DNA in triplicates, corresponding to 10.0 ng, 5.0 ng, 1.0 ng, 0.5 ng, 0.1 ng, 50.0 pg, 10.0 pg, 5.0 pg, 1.0 pg, and 0.5 pg (6.8×10^7 to 6.8 AgMNPV genome copies), were amplified using standard conditions already described for PCR of the polyhedrin region. The intensities of the bands corresponding to the amplified products for the competitor and target DNA were quantified by volume integration using a PhosphorImager. These values were entered in the Excel spreadsheet and the ratios of the amounts of target PCR product to that of the competitor PCR product, and their logarithms were calculated and subsequently plotted as a function of the logarithm of the input amount of target DNA to obtain a regression line. The standard curve was calculated in Excel by a least-squares analysis (Lee et al., 1996) to determine the amount of AgMNPV DNA in field-collected

soil samples by relating the ratio of the amount of the amplification product of the unknown sample to that of the target DNA in the standard curve.

Competitive PCR Product Visualization and Analysis

NuSieve gel electrophoresis and alkaline blotting

Conditions for the polyhedrin competitive PCR are described in Appendix J. All the amplification products for the cPCR were analyzed by 3% NuSieve GTG 3:1 (FMC, Inc.) gel electrophoresis in TAE buffer stained with ethidium bromide. DNA in the gels was alkaline blotted to Zeta-Probe membranes (Bio-Rad, Inc., Hercules, CA), according to the manufacturer's instructions (Appendix K). The blotted membranes were air-dried, wrapped in Whatman Chromatography paper, and baked at 80°C for 30 min.

Prehybridization, hybridization, and washes

These procedures were carried out according to Bio Rad's instructions in a Mini Hybridization Oven. The membranes were prehybridized for two hours at 65°C in 1 mM EDTA, 0.5 M NaH₂PO₄ pH 7.2, 7% SDS. DNA on the blotted membranes was hybridized overnight at 65°C to a ³²P radiolabeled 297 bp AluI fragment within the *polyhedrin* coding region (+317 to +614). This fragment is 100% homologous to the target and competitor polyhedrin PCR product. To make the probe, 100 ng of the 297 AluI DNA fragment was labeled with ³²P-dCTP using nick translation with minimal DNase procedure (modified from the USB Nick Translation Protocol-Appendix L). The membranes were hybridized for 16 hours at 65°C in 10 ml fresh prehybridization buffer containing the radiolabeled probe. After hybridization, the membranes were washed four times for 30 min each in 1 mM EDTA, 40 mM NaHPO₄ pH 7.2, 5% SDS (two washes),

or 1% SDS (last two washes). After the washings, the membranes were sealed in plastic bags and exposed to PhosphorImager cassettes overnight in the dark at room temperature.

PhosphorImager analysis

The exposed cassettes containing the resulting DNA images were scanned in a PhosphorImager (Molecular Dynamics, Inc.), and the separated bands, corresponding to amplified target and competitor DNAs, were quantified by volume integration using ImageQuant software v 3.0 (Molecular Dynamics, Inc.). The values obtained from the volume integration were entered in Excel, and the ratios as well as the logarithms of the ratios between AgMNPV polyhedrin target and competitor PCR products were calculated.

Efficiency of DNA Isolation Procedure

We used cPCR to determine the efficiency of the MCH procedure for isolation of AgMNPV DNA from soil. Soil samples, in triplicates, were inoculated with 10^9 , 10^8 , 10^7 , 10^6 , and 10^5 AgMNPV polyhedra per 0.25 g of soil. This experiment was performed on two separate occasions. AgMNPV DNA was extracted by the MCH procedure, and cPCR was performed with either 2.62×10^7 (100 pg) or 1.31×10^7 (50 pg) plasmid copies of competitor DNA.

Aliquots of AgMNPV DNA purified from virions and corresponding to a range of 10^9 to 10^5 AgMNPV polyhedra (same amounts of polyhedra inoculated in the soil samples) served as controls, and were used directly for cPCR amplification with 100 and 50 pg of competitor DNA. Two calibration curves were constructed from these controls, one for each amount of competitor DNA used for cPCR. Regression equations were

generated from these calibration curves and were used to calculate the initial amounts of target DNA in the soil samples after the DNA extraction procedure. The regression equations were $y=0.7547x + 0.5207$ and $y=0.4275x + 0.5056$ for 100 and 50 pg of competitor DNA, respectively.

PCR products were visualized on 3% NuSieve gels, and alkaline-blotted to Zeta-Probe membranes, as described before. The membranes were hybridized to the ^{32}P AluI polyhedrin probe, exposed to a PhosphorImager cassette, and the intensity of the resultant bands was calculated with the aid of the ImageQuant software v3.0. The efficiency of the MCH procedure should be obtained by a comparison between DNA copies present in the controls and in the soil samples.

Results

Sensitivity of DNA Extraction Procedures

The quantity of DNA present in a known number of AgMNPV polyhedra was calculated according to Volkman et al. (1976) and Volkman & Summers (1977). In those studies, the DNA-protein ratio of AcMNPV preparations was determined using the diphenylamine method (for DNA values) and the Lowry method (for protein values). The number of AcMNPV genome copies obtained per polyhedron was 36. The average number of nucleocapsids per envelope and the average number of envelope-nucleocapsids per polyhedra was determined by examining photographs of TEM thin sections (90,000 x) of AcMNPV and AgMNPV. The number of nucleocapsids per envelope was multiplied by the number of envelope-nucleocapsids per polyhedra, for

both viruses, to obtain the total number of genome equivalents per polyhedron. The ratio between the total numbers found for AcMNPV and AgMNPV was multiplied by 36 (AcMNPV genome copies/polyhedron; Volkman & Summers, 1977). According to this procedure, the number of AgMNPV genome copies obtained per polyhedron was 28, and this number was used to calculate the approximate number of AgMNPV genome copies from known amounts of polyhedra, as described above.

The phenol-ether procedure extracted humic acids from soil by sequential incubation in sodium pyrophosphate and sodium hydroxide, and then purified the viral DNA by successive phenol-ether extractions. Baculovirus DNA extraction using this method removed a large amount of humic acids from soil, but there was still some inhibition during PCR amplification. Therefore, DNA had to be diluted 10 or 100 times to be used in PCR experiments. A detection limit experiment repeated on three separate occasions showed that the limit of detection for this procedure ranged from 1.1×10^4 (two experiments) to 1.1×10^5 (1 experiment) AgMNPV genome copies per gram of soil which corresponds to 4×10^2 to 4×10^3 polyhedra per gram of soil, respectively. Figure 4.1 shows the results of one of these experiments in which 4×10^2 polyhedra per gram of soil was the lowest amount detected (lane 28). The MCH procedure used to isolate AgMNPV DNA from soil was efficient in removing humic acids and other inhibitors. Therefore, DNA aliquots were added directly in PCR amplification without dilution. Four replications of the same experiment determined that the detection limit for this procedure was 1.1×10^2 to 1.1×10^3 genome copies per gram of soil which is equivalent

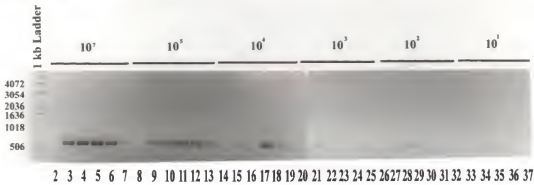


Figure 4.1: Detection Limit of the phenol-ether procedure to isolate AgMNPV DNA from soil. Autoclaved soil (0.25 g) was inoculated with AgMNPV polyhedra, the viral DNA was extracted by the phenol-ether method and PCR-amplified for the *polyhedrin* gene. All bands correspond to the 575 bp polyhedrin PCR product. Lane 1: Molecular marker (1 kb Ladder); lanes 2-4: 10^7 polyhedra/0.25 g of soil. DNA extracts were undiluted (lane 2), 10 x (3), and 100 x diluted (4); lanes 5-7: 10^7 polyhedra with no soil added. DNA extracts were undiluted (lane 5), 10 x (6), and 100 x diluted (7); lanes 8-10: 10^5 polyhedra/0.25 g of soil. DNA extracts were undiluted (lane 8), 10 x (9), and 100 x diluted (10); lanes 11-13: 10^5 polyhedra with no soil added. DNA extracts were undiluted (lane 11), 10 x (12), and 100 x diluted (13); lanes 14-16: 10^4 polyhedra/0.25 g of soil. DNA extracts were undiluted (lane 14), 10 x (15), and 100 x diluted (16); lanes 17-19: 10^4 polyhedra with no soil added. DNA extracts were undiluted (lane 17), 10 x (18), and 100 x diluted (19); lanes 20-22: 10^3 polyhedra/0.25 g of soil. DNA extracts were undiluted (lane 20), 10 x (21), and 100 x diluted (22); lane 23-25: 10^3 polyhedra with no soil added. DNA extracts were undiluted (lane 23), 10 x (24), and 100 x diluted (25); lanes 26-28: 10^2 polyhedra/0.25 g of soil. DNA extracts were undiluted (lane 26), 10 x (27), and 100 x diluted (28); lanes 29-31: 10^2 polyhedra with no soil added. DNA extracts were undiluted (lane 29), 10 x (30), and 100 x diluted (31); lanes 32-34: 10^1 polyhedra/0.25 g of soil. DNA extracts were undiluted (lane 32), 10 x (33), and 100 x diluted (34); lanes 35-37: 10^1 polyhedra with no soil added. DNA extracts were undiluted (lane 35), 10 x (36), and 100 x diluted (37).

to 4 and 40 polyhedra per gram of soil, respectively. The lowest limit of detection, 4 polyhedra per gram of soil, was obtained in three of the four experiments performed, and the results for one of these experiments are shown in Figure 4.2 (lane 9). The MCH procedure was a 100 fold more sensitive than the phenol-ether extraction. Therefore, MCH was the method used to detect AgMNPV in field soil samples.

Detection of AgMNPV DNA from Field Soil Samples

Magnetic capture-hybridization followed by PCR amplification of the *polyhedrin* gene did not detect AgMNPV DNA in soil samples collected from control plots and AgMNPV-2D treatments one day after virus application. In contrast, AgMNPV DNA was detected in 70%, 100%, 80%, and 80% of soil samples collected from the AgMNPV-2D treatments at 15, 45, 75, and 180 days post-application, respectively. AgMNPV DNA was not detected in soil from control plots 15 days post-application, but it was detected in 40%, 20%, and 10% of the soil samples from control plots collected at 45, 75, and 180 days post-application, respectively. No viral DNA was detected from soil samples collected 330 days post-application (Figure 4.3).

Competitive PCR

The competitor DNA had the same nucleotide sequence as the *polyhedrin* gene target DNA, except for a deletion of 65 bp inside the *polyhedrin* coding region (+252 to +317). The primer binding sites were the same for wild-type and competitive DNA sequences, and the competitor PCR product was 510 bp long. Therefore, both polyhedrin target (575 bp) and competitor (510 bp) PCR products were distinguished by NuSieve gels on the basis of their difference in size.

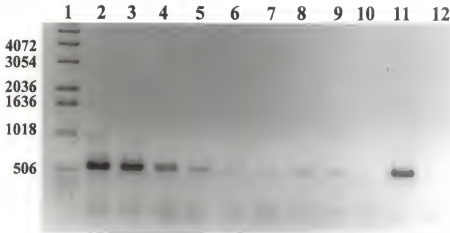


Figure 4.2: Detection limit of the magnetic capture-hybridization procedure to isolate AgMNPV DNA from soil. Autoclaved soil (0.25 g) was inoculated with AgMNPV polyhedra, the viral DNA was extracted by the MCH procedure and PCR-amplified for the *polyhedrin* gene. Lane 1: Molecular marker (1 kb Ladder); lanes 2-9: 575 bp polyhedrin PCR product corresponding to 10^7 , 10^7 (no soil), 10^5 , 10^4 , 10^3 , 10^2 , 10^1 , and 1 polyhedra per 0.25 g of soil, respectively; lane 10: Negative control, corresponding to soil with no virus added; lane 11: Positive control, corresponding to AgMNPV DNA purified from virions; lane 12: Reagents control, corresponding to all PCR reagents with no DNA added.

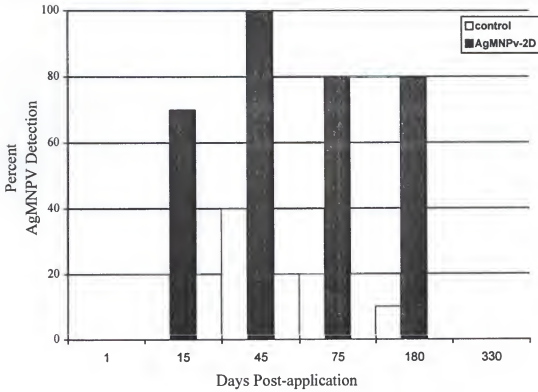


Figure 4.3: Percent PCR detection of the *Anticarsia gemmatalis* nucleopolyhedrovirus in soil samples. Percentage values correspond to the total number of soil samples collected per treatment in each sampling date. PCR amplification targeted the *polyhedrin* gene.

Data from the PhosphorImager analysis showed that the PCR amplification efficiency for the competitor DNA averaged 0.94 times the PCR amplification efficiency for the AgMNPV polyhedrin target DNA (Figure 4.4). The test for heteroduplex formation, in which amplified mixtures of competitor and target DNA were heated at 95°C for 5 min, only produced two bands of the expected size (510 and 575 bp, respectively). This indicated that there was no heteroduplex formation (Figure 4.5).

A standard curve was generated by amplifying a range of amounts of AgMNPV polyhedrin target DNA, corresponding to 10.0 ng, 5.0 ng, 1.0 ng, 0.5 ng, 0.1 ng, 50.0 pg, 10.0 pg, 5.0 pg, 1.0 pg, and 0.5 pg (6.8×10^7 to 6.8 genome copies) in the presence of a constant amount of 1.0 pg of competitor DNA (2.62×10^5 plasmid copies). Figure 4.6 A shows the resulting bands on a NuSieve gel for cPCR of the *polyhedrin* gene. PCR products for both, AgMNPV polyhedrin target DNA and competitor DNA, were observed only for amounts of target DNA between 10 ng and 10 pg. The resulting bands on the gel were blotted to a nitrocellulose membrane, hybridized to a ^{32}P 297 bp AluI probe, and exposed to a PhosphorImager cassette. The DNA images corresponding to amplified target DNA (Figure 4.6B). The resulting regression equation for this standard curve was $y = 0.5363x + 0.4113$ with an $R^2 = 0.9163$, where “y” corresponds to the logarithm of the target/competitor ratio and “x” corresponds to the logarithm of the input amount of target DNA. To validate the quantification system, the log ratios of the target PCR product and competitor PCR product that had been used to obtain the calibration curve were entered in the regression equation to calculate the initial amount of target DNA (Table 4.1).

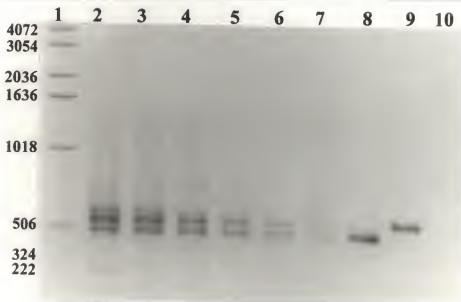


Figure 4.4: PCR amplification efficiency test. A mixture of equal concentrations of competitor and target PCR products were diluted 1 in 10^6 , and $1\ \mu\text{l}$ aliquots were reamplified. Lane 1: Molecular marker (1 kb Ladder); lanes 2-7: mixture of target (575 bp) and competitor (510 bp) polyhedrin PCR product, corresponding to 10 ng, 1 ng, 0.1 ng, 10 pg, 1 pg, and 0.1 pg of PCR product mixture /25 μl ; lane 8: competitor PCR product (510 bp); lane 9: target PCR product (575 bp); lane 10: Reagents control (all PCR reagents added, except template DNA). Ratio of competitor to target was determined after amplification using a PhosphorImager (Molecular Dynamics, Inc.).

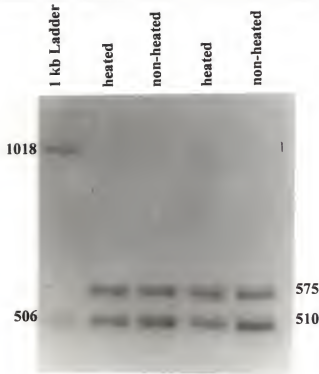


Figure 4.5: Test for heteroduplex formation. Duplicates of equal mixtures of competitor and target polyhedrin PCR products were treated by heating to 95°C for 5 min (lanes 2 and 4). Controls corresponded to non-heated mixtures (lanes 3 and 5). Target polyhedrin PCR product is 575 bp; competitor PCR product is 510 bp. Lane 1: Molecular marker (1 kb Ladder). The treated and non-treated mixtures were electrophoresed on 2% Seakem LE agarose gel.

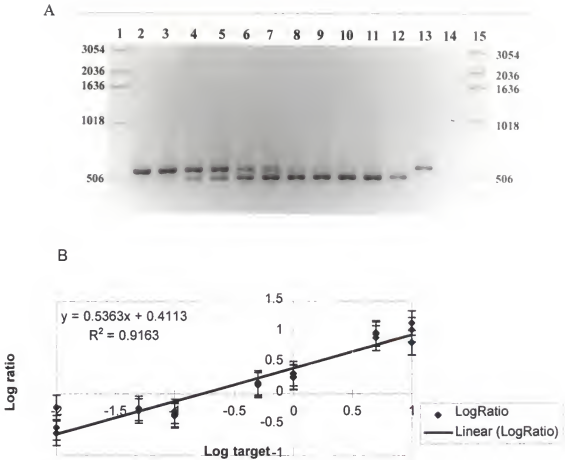


Figure 4.6: (A) Range of amounts of target DNA (AgMNPV DNA) co-amplified with 1 pg of competitor DNA. Lanes 1 and 15: Molecular marker (1 kb Ladder); lanes 2- 11: cPCR products for the *polyhedrin* gene corresponding to 10 ng, 5 ng, 1 ng, 0.5 ng, 0.1 ng, 50 pg, 10 pg, 5 pg, 1.0 pg, and 0.5 pg of input AgMNPV target DNA, respectively; lane 12: polyhedrin PCR product for the 510 bp competitor DNA; lane 13: polyhedrin PCR product for AgMNPV DNA; lane 14: Reagents control (all PCR reagents were added to the reaction, except the template DNA). The ratios between target and competitor PCR product were calculated using the PhosphorImager (Molecular Dynamics, Inc.) and used to create the calibration line on panel B. (B) Standard curve for competitive PCR of the AgMNPV *polyhedrin* gene. The logarithm of the ratio of the amount of the target PCR product to that of the competitor PCR product was plotted against the logarithm of the initial amount of target DNA. PCR band intensities were analyzed by the ImageQuant software v 3.0. Regression equation and R^2 are shown in the chart. Standard deviation is shown as error bars.

Table 4.1: Validation of the standard curve using 1.0 pg of competitor DNA. Calculated target concentrations were obtained using the regression equation displayed in Figure 4.6 B ($y = 0.5363x + 0.4113$), where “y” corresponds to the logarithm of the target/competitor ratio and “x” corresponds to the logarithm of the input amount of target DNA. The log ratio values were substituted in the equation and resulted in log target values. The log target values were transformed to absolute numbers and multiplied by 0.94 (amplification efficiency of the competitor DNA in relation to target DNA).

Input target (in ng)	Log target	Target/Competitor	Log ratio	Calculated log target	Calculated target (in ng)
10.0	1.0	6.6615	0.8235	1.1243	12.5168
10.0	1.0	10.8075	1.0337	1.5162	30.8561
10.0	1.0	13.9605	1.1449	1.7236	49.7496
5.0	0.6989	9.2353	0.9654	1.3889	23.0165
5.0	0.6989	9.7139	0.9873	1.4298	25.2902
5.0	0.6989	7.9195	0.8987	1.2644	17.2810
1.0	0	2.1111	0.3245	0.1938	1.4687
1.0	0	1.8617	0.2699	0.0920	1.1617
1.0	0	1.8266	0.2616	0.0766	1.1213
0.5	-0.3010	1.4140	0.1504	-0.1307	0.6956
0.5	-0.3010	1.3722	0.1374	-0.1550	0.6578
0.5	-0.3010	1.5017	0.1765	-0.0820	0.7782
0.1	-1.0	0.5159	-0.2873	-0.9471	0.1061
0.1	-1.0	0.4586	-0.3385	-1.0424	0.0852
0.1	-1.0	0.4372	-0.3592	-1.0811	0.0779
0.05	-1.3010	0.5242	-0.2804	-0.9342	0.1093
0.05	-1.3010	0.5764	-0.2392	-0.8574	0.1305
0.05	-1.3010	0.5226	-0.2818	-0.9368	0.1087
0.01	-2.0	0.2269	-0.6441	-1.6123	0.02295
0.01	-2.0	0.2800	-0.5526	-1.4418	0.03398
0.01	-2.0	0.5967	-0.2241	-0.8293	0.1392

There was a good correlation between “real” and “calculated” initial amounts of target DNA within a range of 1 and 0.1 ng of input target DNA, corresponding to 10^6 to 10^5 copies ($R^2 = 0.9415$) (Figure 4.7). Amounts of target DNA above 1.0 ng or below 0.1 ng did not have a linear response, and therefore, were considered out of the quantitative range of this system (data not shown).

Quantitation of AgMNPV DNA from soil samples collected in the field was attempted by co-amplifying aliquots of the unknown samples with constant amounts of 1.0 or 0.1 pg of the 510 bp competitor DNA, corresponding to 10^5 and 10^4 plasmid copies, respectively. When cPCR was performed with 1.0 pg of competitor DNA, the only amplified product corresponded to the competitor product (data not shown). This indicated that the number of genome copies present in the soil was much lower than the number of competitor copies. When cPCR was performed with 0.1 pg of competitor DNA, target and competitor PCR products were observed for some samples collected 45, 75 and 180 days post-application in the AgMNPV-2D treated plots (Figure 4.8). However, the bands corresponding to the PCR products for the AgMNPV polyhedrin target DNA were much less intense than the bands corresponding to the competitor PCR products.

Efficiency of the MCH Procedure

Accurate DNA quantitation by cPCR must take into account the efficiency of the DNA isolation procedure. We used a cPCR approach to determine the efficiency of the MCH procedure in extracting AgMNPV DNA from soil.

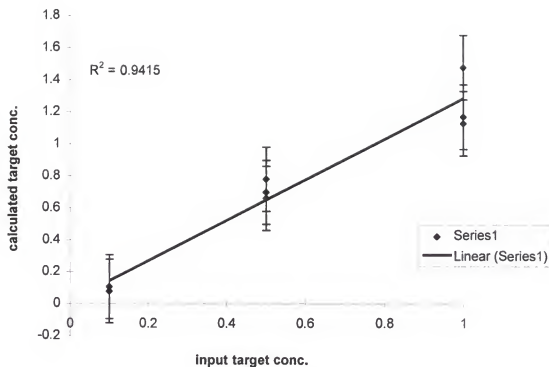


Figure 4.7: Accuracy of AgMNPV DNA quantitation by competitive PCR. Calculated target concentrations in nanograms were obtained using the regression equation displayed in Figure 4.6-B. The calculated concentrations were plotted against actual input concentration of target DNA in nanograms. Standard deviation is shown as error bars. R^2 is shown in the chart.

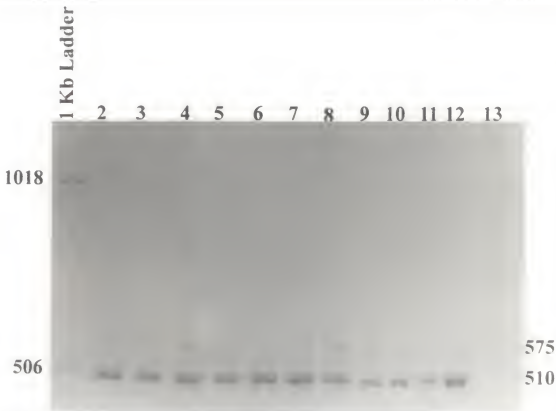


Figure 4.8: Competitive polyhedrin PCR for field-collected soil samples. Soil samples were collected at 45 (lanes 2-5), 75 (lanes 6-9), and 180 (lanes 10 and 11) days after AgMNPV application. The amount of competitor DNA per PCR reaction was 0.1 pg. Lane 12 : positive control (1.0 pg of AgMNPV DNA purified from virions and 0.1 pg of competitor DNA). Lane 13: reagents control (all the PCR reagents, except template DNA). The molecular weight standard used was the 1 Kb Ladder (Lane 1).

Two experiments were performed in which autoclaved soil was inoculated, in triplicates, with 10^9 , 10^8 , 10^7 , 10^6 , and 10^5 AgMNPV polyhedra per 0.25 g of soil, corresponding to 10^8 to 10^3 AgMNPV genome copies. The extracted DNA was co-amplified with either 100 pg (1.31×10^7 copies) or 50 pg of competitor DNA (2.62×10^7 copies). These concentrations of competitor DNA were used because co-amplification with 1.0 and 0.1 pg of competitor DNA (concentrations that had been used for cPCR from field-collected samples) did not produce amplifiable products for the amounts of polyhedrin target DNA used in these experiments.

Partial results from experiment 1 are shown in Figure 4.9 in which the resulting PCR product bands for the AgMNPV polyhedrin target DNA were co-amplified with 100 pg of the 510 bp competitor DNA. The bands on the NuSieve gel appear to be uniform for the replicates corresponding to the same amounts of target DNA. An exception was the target and competitor PCR products corresponding to soil samples inoculated with 10^9 polyhedra, in which each of the three replicates presented a different result (lanes 2-4). The differences in band intensities for replicates of target and competitor PCR products when the soil samples were inoculated with 10^9 polyhedra, could be attributed to human error during the DNA isolation procedure or PCR set up. Another possible explanation is that recovery of AgMNPV DNA from soil was not uniform for the DNA isolation procedure developed in this study. NuSieve gel results of cPCR of the *polyhedrin* gene co-amplified with 50 pg of competitor DNA are not shown for experiment 1.

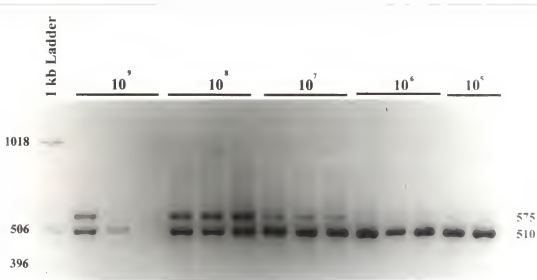


Figure 4.9: cPCR results for AgMNPV DNA isolated from soil using the MCH procedure. Autoclaved soil was inoculated, in triplicates, with 10^9 (lanes 2-4), 10^8 (lanes 5-7), 10^7 (lanes 8-10), 10^6 (lanes 11-13), and 10^5 (lanes 14 and 15) AgMNPV polyhedra per 0.25 g of soil. The DNA was extracted by magnetic capture-hybridization and co-amplified with 100 pg of competitor DNA. Resulting cPCR product bands were visualized by ultraviolet light on a 3% NuSieve gel. Lane 1: Molecular marker (1 kb Ladder).

NuSieve gel results are not shown for experiment 2. In experiment 2, the results are presented as target/competitor ratios and their logarithms, which were generated from the PhosphorImager analysis for cPCR of the *polyhedrin* gene using either 100 pg or 50 pg of competitor DNA (Tables 4.2 and 4.3). The PhosphorImager analysis revealed larger variability among replicates within the same DNA copy number than observed in the previous experiment with 100 pg of competitor DNA (Table 4.2). A comparison of the data presented in Figure 4.9 and Table 4.2 reveals inter assay variability. For example, in experiment 1, a similar ratio between polyhedrin target and competitor PCR products was observed when the soil was inoculated with 10^9 polyhedra per 0.25 g of soil (lane 2 of Figure 4.9). However, in experiment 2, the PhosphorImaging analysis revealed a target/competitor ratio around 9.0, when the soil was inoculated with 10^9 polyhedra per 0.25 g of soil (Table 4.2). This indicates that the polyhedrin target PCR product yield was 9 times higher than the competitor PCR product yield. Similarly, the amounts of target/competitor ratios differed in the two experiments when the soil was inoculated with 10^8 polyhedra per 0.25 g of soil. In experiment 1, the target/competitor ratios appear to be around 1.0 (Figure 4.9; lanes 5-7). In experiment 2, the PhosphorImager analysis determined a target/competitor ratio of 2.9 and 2.4 for the same amount of viral polyhedra inoculated in the soil (Table 4.2). Table 4.3 also shows some variability among replicates for the ratios between AgMNPV polyhedrin target DNA and 510 bp competitor DNA, when cPCR was performed with 50 pg of competitor DNA. A comparison of the data

Table 4.2: Ratios between AgMNPV polyhedrin target and 510 bp competitor PCR products and "calculated" target concentrations using 100 pg of competitor DNA. A series of amounts of target DNA was co-amplified with 100 pg of competitor DNA. The ratios between target and competitor PCR products were calculated from the PhosphorImager analysis; the log target was obtained by entering the log ratio data in the following regression equation: $y = 0.7547x + 0.5207$ (Figure 4.10) where "y" corresponds to the logarithm of the target/competitor ratio and "x" corresponds to the logarithm of the input amount of target DNA.

Virus Conc.	Theoretical Amount of AgMNPV DNA in ng.	Target/Competitor	Log ratio	Log target	Calculated Target (in ng)
10^9	5000	9.1329	0.9606	2.6957	496.2921
10^9	5000	8.5817	0.9335	2.6093	406.7826
10^8	500	2.9646	0.4719	1.1345	13.6323
10^8	500	2.4505	0.3892	0.8703	7.4186
10^7	50	1.7609	0.2457	0.4117	2.5810
10^7	50	1.0358	0.0152	-0.3245	0.4737
10^7	50	0.9541	-0.0204	-0.4385	0.3643
10^6	5.0	0.2144	-0.6687	-2.5099	0.0030
10^5	0.5	0.0914	-1.0388	-3.6922	0.0002

Table 4.3: Ratios between AgMNPV polyhedrin target and 510 bp competitor PCR products and "calculated" target concentrations using 50 pg of competitor DNA. A series of amounts of target DNA was co-amplified with 50 pg of competitor DNA. The ratios between target and competitor PCR products were calculated from the PhosphorImager analysis; the log target was obtained by entering the log ratio data in the following regression equation: $y = 0.4275x + 0.5056$ (Figure 4.11) where "y" corresponds to the logarithm of the target/competitor ratio and "x" corresponds to the logarithm of the input amount of target DNA.

Virus Conc.	Theoretical Amount of AgMNPV DNA in ng.	Target/Competitor	Log ratio	Log target	Calculated Target (in ng)
10^9	5000	0.5989	-0.2226	-0.8821	0.1311
10^9	5000	0.3903	-0.4086	-1.6346	0.0231
10^8	500	0.5756	-0.2398	-0.9518	0.1117
10^8	500	0.5226	-0.2818	-1.1216	0.0755
10^7	50	0.4143	-0.3826	-1.5298	0.0295
10^7	50	0.3888	-0.4102	-1.6414	0.0228
10^7	50	0.2311	-0.6362	-2.5557	0.0027
10^6	5	0.1155	-0.9374	-3.7747	0.0001
10^6	5	0.4123	-0.3847	-1.5383	0.0289
10^5	0.5	0.3287	-0.4832	-1.9365	0.0115
10^5	0.5	0.3664	-0.4360	-1.7457	0.0179
10^5	0.5	0.1106	-0.9562	-3.8509	0.0001

presented in Tables 4.2 and 4.3 shows that the target/competitor ratios and the calculated polyhedrin target DNA were not similar for most of the observations when cPCR was performed with different concentrations of competitor DNA.

Two standard curves were constructed using either 100 pg (Figure 4.10) or 50 pg (Figure 4.11) of competitor DNA and a range of target DNA concentrations obtained from AgMNPV DNA purified from virions. The regression equation for the polyhedrin cPCR standard curve using 100 pg of competitor DNA ($y = 0.7547x + 0.5207$; $R^2 = 0.9938$) was obtained from only two input amounts of target DNA, corresponding to 0.5 and 0.05 ng (Figure 4.10). PCR products corresponding only to the 510 bp competitor DNA were observed for cPCR amplification of the *polyhedrin* gene using the other amounts of input target DNA. This equation was used to calculate the initial amounts of polyhedrin target DNA after DNA isolation from soil using the MCH procedure (Table 4.2). The same equation was used to validate the standard curve results by substituting the log ratio values in the equation (y) and obtaining calculated log target values (x) which were transformed to absolute numbers and multiplied by the PCR amplification efficiency of the 510 bp competitor DNA in relation to the PCR amplification efficiency of the polyhedrin target DNA (0.94) (Table 4.4).

Figure 4.11 shows the regression equation for the polyhedrin cPCR standard curve using 50 pg of competitor DNA ($y = 0.4275x + 0.5056$; $R^2 = 0.9448$). This regression equation was calculated using a broader range of target DNA amounts (5.0, 0.5, 0.05, and 0.005 ng of target DNA per PCR reaction), and it was used to calculate the initial amounts of polyhedrin target DNA after MCH DNA isolation from soil and cPCR

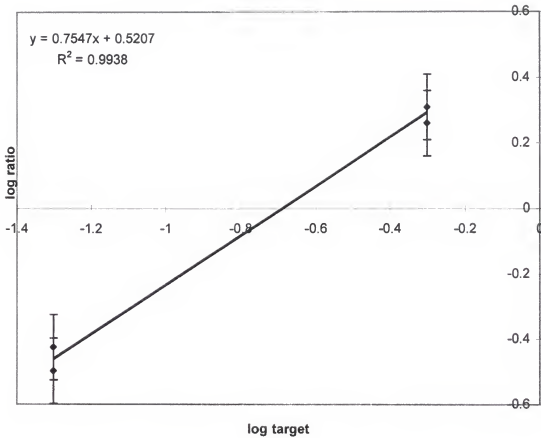


Figure 4.10: Standard Curve of competitive PCR of AgMNPV *polyhedrin* gene using 100 pg of competitor DNA. A series of amounts of target DNA were co-amplified with 100 pg of competitor DNA. The logarithm of the ratio of the amount of the target PCR product to that of the competitor PCR product (y) was plotted against the logarithm of the initial amount of target DNA (x). PCR band intensities were analyzed by the ImageQuant software v 3.0. Regression equation and R^2 are shown in the chart. Standard deviation is shown as error bars. The resulting regression equation obtained in this standard curve was used to calculate amounts of target DNA in Table 4.2.

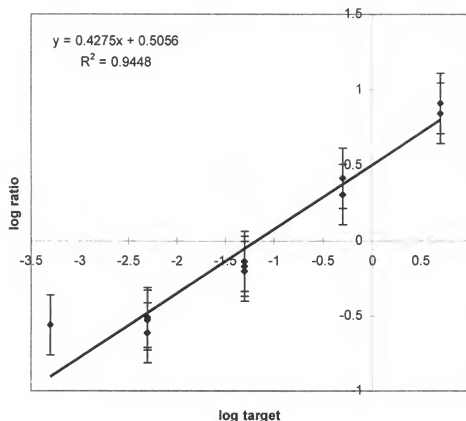


Figure 4.11: Standard curve of competitive PCR of AgMNPV *polyhedrin* gene using 50 pg of competitor DNA. A series of amounts of target DNA were co-amplified with 50 pg of competitor DNA. The logarithm of the ratio of the amount of the target PCR product to that of the competitor PCR product (y) was plotted against the logarithm of the initial amount of target DNA (x). PCR band intensities were analyzed by the ImageQuant software v 3.0. Regression equation and R^2 are shown in the chart. Standard deviation is shown as error bars. The resulting regression equation obtained in this standard curve was used to calculate amounts of target DNA in Table 4.3.

Table 4.4: Validation of the standard curve using 100 pg of competitor DNA. Calculated target concentrations were obtained using the regression equation displayed in Figure 4.10 ($y = 0.7547x + 0.5207$). The log ratio values (y) were substituted in the equation and resulted in log target values (x). The log target values were transformed to absolute numbers and multiplied by 0.94 (amplification efficiency of the competitor DNA in relation to target DNA).

Input target (in ng)	Log target	Target/Compet itor	Log ratio	Calculated log target	Calculated target (in ng)
0.5	-0.3010	2.0409	0.3098	0.9312	8.0235
0.5	-0.3010	2.0409	0.3098	0.9312	8.0235
0.5	-0.3010	1.8228	0.2607	0.8662	6.9076
0.05	-1.3010	0.3758	-0.4249	-0.0424	0.8525
0.05	-1.3010	0.3180	-0.4975	-0.1385	0.6832

Table 4.5: Validation of the standard curve using 50 pg of competitor DNA. Calculated target concentrations were obtained using the regression equation displayed in Figure 4.11 ($y = 0.4275x + 0.5056$). The log ratio values (y) were substituted in the equation and resulted in log target values (x). The log target values were transformed to absolute numbers and multiplied by 0.94 (amplification efficiency of the competitor DNA in relation to target DNA).

Input target (in ng)	Log target	Target/Compet itor	Log ratio	Calculated log target	Calculated target. (in ng)
5.0	0.6989	8.1453	0.9109	1.6251	39.6557
5.0	0.6989	8.1521	0.9112	1.6260	39.7336
5.0	0.6989	7.0091	0.8456	1.4725	27.9058
0.5	-0.3010	2.6074	0.4162	0.4680	2.7615
0.5	-0.3010	2.0263	0.3067	0.2118	1.5310
0.05	-1.3010	0.6775	-0.1690	-0.9010	0.1180
0.05	-1.3010	0.7305	-0.1363	-0.8245	0.1407
0.05	-1.3010	0.6280	-0.2019	-0.9780	0.0988
0.005	-2.3010	0.2973	-0.5266	-1.7375	0.0172
0.005	-2.3010	0.2441	-0.6123	-1.9379	0.0108
0.005	-2.3010	0.3096	-0.5091	-1.6966	0.0188
0.0005	-3.3010	0.2757	-0.5594	-1.8142	0.0144

using 50 pg of competitor DNA (Table 4.3). This equation was also used to validate the standard curve results when polyhedrin cPCR was performed with 50 pg of competitor DNA (Table 4.5). The results displayed in Tables 4.4 and 4.5 showed that the “calculated” target amounts were not similar to the input polyhedrin target DNA amounts. Therefore, our attempts to determine the efficiency of the DNA isolation procedure were not uniform, and quantitation of AgMNPV DNA from soil was not validated in our system.

Discussion

In this study, two different methods were developed and optimized to extract baculovirus DNA directly from soil, which were suitable for PCR amplification. The better method, magnetic capture-hybridization, detected as low as 110 AgMNPV genome copies per gram of soil (4 polyhedra/g of soil), while the method using phenol-ether extractions detected 1.1×10^4 to 1.1×10^5 genome copies per gram of soil (4×10^2 to 4×10^3 polyhedra/g of soil, respectively). The apparent inhibitory effect of humic acids was completely removed by the MCH procedure, since direct aliquots of the DNA preparations could be amplified without dilutions. In contrast, the phenol-ether method apparently did not remove humic acids completely, because 10 to 100 fold dilution of DNA was necessary to obtain PCR amplification. These results indicate that the MCH procedure is better suited for baculovirus DNA extraction from soil than the phenol-ether method. However, the MCH method is more costly than the phenol-ether procedure.

The MCH procedure coupled with PCR amplification of the *polyhedrin* gene was more sensitive than ELISA and radioimmunoassay techniques. Naser & Miltenburger

(1983) detected 3×10^5 AcMNPV polyhedra using monoclonal antibodies, while Crawford et al. (1977) detected 5×10^3 and 1.2×10^3 polyhedra of *Wiseana* NPV using a direct and indirect radioimmunoassay, respectively. DNA techniques such as dot blot hybridization were less sensitive than our method, since detection levels between 10^5 to 10^6 genome copies were reported (Ward et al., 1987; Keating et al., 1989; Kaupp & Ebling, 1993; Kukan & Myers, 1995). Many of the detection techniques discussed above were limited by the use of radioisotopes, which increases safety concerns. In addition, they detected baculovirus polyhedra or DNA from larval homogenates instead of soil. Polyhedra or viral DNA extraction from soil is challenging due to the strong adsorption of polyhedra to positively charged particles in the soil (Hukuhara & Wada, 1972), and due to the presence of humic acids and other inhibitory compounds that inhibit PCR amplification (Tsai & Olson, 1992; Tebbe & Vahjen, 1993).

Neonate bioassays have detected 4×10^4 SfNPV polyhedra/g in sandy soils (45% sand) and 10 polyhedra/g in soils with high content of silt or clay (Fuxa et al., 1985). In a subsequent study, Fuxa & Richter (1993) detected 16 AgMNPV polyhedra/g in a soil containing 2.4% sand, 76.0% silt, and 21.6% clay, and 318 polyhedra/g in a soil composed of 25.0% sand, 54.0% silt, and 20.9% clay. Wood et al. (1994) also have used bioassays with neonates, and detected 7 AcMNPV polyhedra/g of soil, however, soil type was not mentioned. It appears that the sensitivity of baculovirus detection in the soil is inversely correlated with the sand content; more sensitive detection has been associated with low sand contents, while less sensitive detection has been associated with higher sand contents in the soil. In our study, we detected as few as 4 AgMNPV polyhedra/g in

loamy sand soils, containing 70-85% sand. Therefore, the MCH procedure followed by DNA amplification of the polyhedrin region was more sensitive than neonate bioassays for detecting baculoviruses from soil, since the detection levels obtained in soils with intermediate sand content (Fuxa et al., 1985; Fuxa & Richter, 1993) are higher than the levels observed in this study. Our study is the first report on the direct extraction of baculovirus DNA from soil samples.

In the field, AgMNPV DNA was not detected in soil samples one day after virus application. This result was expected since viral application was aimed at the soybean foliage, not at the soil. In addition, there was no larval mortality due to virus disease, and therefore, no polyhedral release from dead larvae one day after virus application. The MCH procedure followed by PCR amplification of the *polyhedrin* gene detected AgMNPV DNA from soil samples collected in a period ranging from 15 to 180 (six months) days post-application. Our method did not detect any virus DNA in control plots or AgMNPV-2D treatments 330 days after virus application. The same virus has been also detected over a period of seven months from soil collected in treated soybean plots in Louisiana (Fuxa & Richter, 1994). Wood et al. (1994) have detected AcMNPV polyhedra from soil over a period of approximately 1.5 years after virus application, using neonate bioassays. Although, the detection levels reported in that study are similar to ours, the virus was detected over a longer period of time. This difference could be attributed to factors such as soil type, which is not mentioned in their study, sample number, subsample number, or sample size, among others. In the present study we detected virus until six months post-application. It is possible that AgMNPV detection

from soil could have been achieved for a longer period of time if we had increased the number of samples or subsample size in our study.

A quantitative approach based on competitive PCR was attempted in the present study to quantify AgMNPV DNA from field-soil samples. The basic requirements for quantitation were achieved. A competitor DNA similar to the target DNA and sharing the same primer binding sites as the target was designed. The competitor DNA was distinguishable from the target DNA in agarose gels by a size difference of 65 bp. In addition, the PCR amplification efficiency for the competitor DNA was 0.94 times that of the AgMNPV polyhedrin target DNA, which was very close to the ideal, where both amplification efficiencies should be equal (McCulloch et al., 1995). In this case, when calculating the concentration of target, the amplification efficiency must be factored into the result. Heteroduplex formation, which is considered a serious limitation to the accurate amplification of DNA or RNA (Ferre, 1992; Hayward-Lester et al., 1996; Zimmermann & Mannhalter, 1996), was not observed for this system.

The quantitation technique developed in this study quantified AgMNPV DNA purified from virions accurately, when the initial input of target DNA was within a range of 1.0 and 0.1 ng per PCR reaction. These AgMNPV DNA preparations were used to construct a standard curve to enable quantitation of field samples. However, quantitation of AgMNPV DNA from field-collected soil samples using cPCR was not achieved mainly due to high inter- and intra-assay variability and narrow quantitation range, which was 1.0 to 0.1 ng of input target DNA per amplification reaction. The isolation of AgMNPV DNA from soil samples might have introduced inter and intra assay variability,

possibly due to the procedure used to disrupt the viral envelope and capsids (boiling of alkali-released virus for 10 min in 1 x PBS buffer). Normally, the disruption of the viral envelope and capsids in baculoviruses is achieved by overnight incubation with Proteinase K followed by several extractions with phenol and ether (Maruniak, 1986). However, one of the goals in this study was to develop a fast and practical protocol to extract baculovirus DNA from environmental samples. Perhaps, the efficiency and uniformity of the disruption of viral envelopes and capsids, in this study, could have been improved by the addition of SDS in the boiling buffer.

Furthermore, according to Ferre (1992), the use of different batches of labeled primers or different tubes of enzyme may alter reproducibility, introducing inter- and intra-assay variability, and therefore, affecting the quantitative power of PCR. In the present study, the competitive DNA used in all experiments was from the same batch, but two different batches of PCR primers, different batches of ^{32}P radiolabeled probe, and different working dilutions of competitor DNA and primers were used. This might have introduced some inter-assay variability. Some additional steps in our cPCR system may have introduced intra-assay variability. For example, it is possible that the blotting of the gels to Zeta-Probe membranes did not occur uniformly, because the bands corresponding to polyhedrin PCR products for target and competitor DNA were close together, even though the gels were run overnight at a voltage of 47 V. The use of polyacrylamide gels, which give better resolution to bands with similar molecular weight, might have been more adequate in this situation. The hybridization step might have introduced more variability to the system. The direct labeling of the PCR products by end-labeling one of

the PCR primers, or by incorporating a radiolabeled dinucleotide to the PCR reaction could be a better approach because it would eliminate the blotting and hybridization steps, since the gels could be dried and exposed directly to the PhosphorImager exposure cassette. Lastly, in the PhosphorImager analysis each band needs to be within a rectangle so that the software program can compute the volume intensity of each band. The rectangles are copied and pasted, using the ImageQuant software, to ensure that they have the same area. However, if the bands are close together, it might be difficult to delineate the bands without introducing errors.

In this study, AgMNPV DNA quantitation from field-collected soil samples was attempted by constructing a standard curve in which a range of amounts of polyhedrin target DNA was co-amplified with a constant amount of 1.0 pg of competitor DNA. However, attempts to quantify unknown AgMNPV DNA from field soil samples by co-amplification with 1.0 pg of the 510 bp competitor DNA produced amplification products corresponding only to the competitor DNA. Therefore, cPCR of the *polyhedrin* gene was attempted using a lower amount (0.1 pg) of competitor DNA, and it produced bands corresponding to the target and competitor amplification products. Nonetheless, the PCR products corresponding to the AgMNPV polyhedrin target DNA were much less intense than the bands corresponding to the competitor PCR products. These results indicated that the number of AgMNPV genome copies present in the soil was much lower than the number of plasmid copies contained in 1.0 pg and 0.1 pg of competitor DNA, which corresponded to 10^5 and 10^4 plasmid copies used for cPCR of the AgMNPV *polyhedrin* gene. It is possible that the higher copy numbers of the competitor DNA outcompeted

the target DNA for dinucleotides, DNA polymerase, and primers during PCR amplification, resulting in none or low PCR product yield for the AgMNPV polyhedrin target DNA.

In theory, a single concentration of competitor DNA co-amplified with the unknown target DNA should be sufficient for quantitation, if the competitor/target ratio is compared to the calibration curve (Jalava et al., 1993). However, our calibration curve was validated only for a narrow range of input target DNA corresponding to 1.0 and 0.1 ng (10^6 - 10^5 copies), limiting the possibilities of quantitation of unknown AgMNPV DNA from field-soil samples. A narrow range of quantitation was also reported by other authors. Ravaggi et al. (1997) developed a cPCR assay in which hepatitis C virus (HCV) quantitation was accurate only within a range of 10^4 to 10^5 HCV RNA copies. However, a direct comparison between their results and my results can not be made because the HCV is an RNA genome, while baculoviruses have a DNA genome. In another study, the quantitation of HIV DNA integrated into the host genome was obtained within a linear range of two orders of magnitude from 10 to 1000 copies (Ferre et al., 1992). In contrast, DNA sequences for the luc gene, which had been inserted into the cyanobacterium *Synechocystis* 6803, were quantitated within a range of 5×10^1 to 1×10^8 copies by using three different calibration curves, with overlapping target DNA concentrations and three fixed concentrations of 10^3 , 10^5 , and 10^7 copies/PCR of competitor DNA (Möller & Jansson, 1997). The construction of overlapping calibration curves as described by Möller & Jansson (1997), could have increased the quantitation range of cPCR in this study. Another aspect that differentiates our study from the studies

discussed above is that we attempted to quantify virus DNA from soil samples, which probably introduced some inter-assay variability and decreased sensitivity of detection. It has been suggested that quantitative PCR should use co-amplification of one concentration of target DNA with several dilutions of competitor DNA (Zimmermann & Mannhalter, 1996), because the quantitative measurements of PCR-amplified products are most accurate when ratios of target and competitor DNA are equal or similar (Raeymaekers, 1993).

In summary, we developed two DNA isolation procedures for direct extraction of baculovirus DNA from soil and subsequent PCR amplification. The magnetic capture-hybridization procedure was more sensitive in extracting AgMNPV DNA from soil than the phenol-ether extraction and other methods such as ELISA, radioimmunoassay, and DNA dot-blot hybridization. The MCH procedure apparently was also more effective in removing humic acids which are usually co-extracted with DNA from soil and are inhibitory to PCR amplification and other enzymatic procedures. This technique enabled detection of AgMNPV DNA from field-soil samples over a six month period. This was the first time that a baculovirus DNA was directly extracted from soil. The development of a cPCR approach enabled quantitation of AgMNPV DNA purified from virions, within a range of 1.0 and 0.1 ng of initial input of polyhedrin target DNA per PCR reaction. However, improvements should be made to enable quantitation of baculovirus DNA from field-soil samples using cPCR. The procedures developed in this research provided a sensitive way to detect baculoviruses from soil, and it should facilitate ecological studies of baculoviruses, since the soil is a major site for persistence of these viruses.

CHAPTER 5

SUMMARY AND DIRECTION OF FUTURE RESEARCH

The detection of baculovirus species and genotypes in the environment was accomplished using the polymerase chain reaction as a detection method. The *polyhedrin* gene was chosen as DNA target for PCR amplification to detect different virus species, because of its highly conserved nature among baculoviruses. A highly variable region, hr4, in the AgMNPV genome, which differs in the number of tandem repeats, was used to detect AgMNPV genotypes.

Eight baculovirus species were detected using PCR amplification of the *polyhedrin* gene including *Autographa californica* multiple nucleocapsid nucleopolyhedrovirus (MNPV), *Anticarsia gemmatilis* MNPV, *Spodoptera frugiperda* MNPV, *S. exigua* MNPV, *Bombyx mori* MNPV, *Orgyia pseudotsugata* MNPV, *Anagrapha falcifera* MNPV, and *Heliothis zea* single nucleocapsid nucleopolyhedrovirus (SNPV). The size of the polyhedrin PCR product was 575 base pairs for all viruses evaluated, which were distinguished by restriction enzyme analysis of the PCR products with HhaI and HincII.

AgMNPV applied in soybean fields was detected from environmental samples including larval hosts, insect predators, and soil. PCR amplification of the *polyhedrin* gene DNA detected AgMNPV in the host and predator populations from one to 45 days after virus application. Two AgMNPV genetic variants, 2D and D7, were applied

separately and as a mixture in the field, and their detection was accomplished by PCR amplification of the hr4 region. The hr4 PCR products were 1,726 and 1,345 base pairs in length for the 2D and D7 genotypes, respectively. AgMNPV was also detected from one to 45 days post-application in field-collected *A. gemmatilis* larvae when the hr4 region was monitored by PCR amplification. However, a McNemar's test for matched proportions demonstrated that PCR of the *polyhedrin* gene was significantly more sensitive in detecting AgMNPV in its larval host than PCR for the hr4 region.

In this study, our primary goals did not include the elucidation of the rate of spread nor the maximum distance of spread of AgMNPV. However, the potential of the PCR technique for dispersal studies was demonstrated by detection of AgMNPV movement from treated plots into the control (PCR for the *polyhedrin* gene), as well as among treated plots (PCR of the hr4 region).

In order to extract AgMNPV DNA from soil samples a phenol-ether extraction and a magnetic capture-hybridization (MCH) procedures were developed and compared in their sensitivity and ability to produce amplifiable DNA. Both procedures produced amplifiable DNA, however, the MCH method was more sensitive than the phenol-ether extraction protocol. Removal of PCR inhibitors from soil appeared to be complete when MCH was used as the AgMNPV DNA isolation method, because undiluted aliquots of the DNA preparations produced amplified products. On the other hand, the phenol-ether procedure probably did not remove PCR inhibitors completely, since PCR products were only observed when the AgMNPV DNA preparations were diluted 10 or 100 times. AgMNPV DNA from field-collected soil samples was detected from 15 to 180 days after

virus application using the MCH procedure to isolate DNA coupled with PCR amplification of the *polyhedrin* gene.

Quantitation of AgMNPV DNA present in field-collected samples was attempted using a competitive PCR (cPCR) approach. The basic requirements for quantitation were achieved. A competitor DNA containing a 65 base pair deletion in comparison to the wild-type *polyhedrin* gene (target DNA) was designed, and both PCR products had the same primer binding sites. The PCR product for the competitive DNA was distinguishable from the PCR product for the polyhedrin target DNA on the basis of size difference. In addition, the PCR amplification efficiency for the competitor DNA was 0.94 times that of the target DNA, which is very close to an ideal situation, in which PCR amplification efficiencies are equal for both products. However, quantitation of AgMNPV DNA by competitive PCR was not validated mainly due to high inter- and intra-assay variability and narrow range of quantitation. Future quantitative studies using cPCR should focus on decreasing variability between and within experiments as well as expanding the range of quantitation.

In summary, environmental detection of baculovirus species and genotypes was accomplished using a conserved and a variable region in the AgMNPV genome as DNA targets for PCR amplification. PCR was a specific, fast, and sensitive way to detect and to identify baculoviruses in the environment. The field tests demonstrated the potential of this detection method to study persistence and dispersal of baculoviruses in the field. The ability to detect baculoviruses at low levels of infection and at early stages of the disease in the field could allow a more dynamic and direct study of their epizootiology.

Furthermore, the procedures developed in this study may be used in quality control of programs in which wild-type or recombinant baculoviruses are being applied to control insects, as well as in quality control of the commercial production of these viruses. The use of the PCR technique as part of the quality control of programs using baculoviruses as microbial control agents would not only monitor persistence and spread of the released virus, but would also determine if that virus is the prevalent virus species or genotype in the field. Similarly, the use of this technique in quality control during the commercial production of baculoviruses would ensure that the desired species and genotypes are prevalent in all batches produced.

The detection methods developed in this research could also be used in risk assessment studies following the release of genetically-improved baculoviruses in the environment. Besides being used to study the persistence and spread of the recombinant virus in the field, PCR could also be used to monitor horizontal transfer of DNA into non-target organisms, to monitor the genetic stability of the inserted DNA, and to detect homologous recombination between the recombinant and native baculoviruses.

In the future, the PCR technique could be expanded to detect a larger number of baculovirus species including granulosis viruses, hymenopteran NPVs, and other lepidopteran MNPVs and SNPVs. This could be accomplished by the design of PCR primers from other conserved regions of the *polyhedrin* gene or from additional conserved baculovirus genes. The gene encoding for the gp41 structural protein could potentially be used as target DNA for PCR amplification with the objective to detect multiple baculovirus species, because its nucleotide sequence was shown to be highly homologous among four baculoviruses that have been sequenced. Expansion of PCR as a

detection method will be possible as more sequences for the *polyhedrin* gene, *gp41* gene, and other baculovirus genes become available. Comparably, the ability to detect genotypes within a particular baculovirus species could be increased by locating and characterizing other variable regions in the genome of these viruses. Detection of baculoviruses from additional environmental samples such as leaves, bird droppings, and water sources would contribute to a better knowledge of the environmental fate of these viruses.

APPENDIX A
PURIFICATION OF POLYHEDRA FROM INFECTED LARVAE

(procedure used in Dr. Maruniak's laboratory).

1. Homogenize infected larvae with the following homogenization buffer:
 - 1% ascorbic acid
 - 2% SDS
 - 0.01 M Tris pH 7.7
 - 0.001 M EDTA pH 8.0
2. Filter in four layers of cheese cloth.
3. Filter through polyester.
4. Centrifuge at 12,000 g for 10 min at 4°C (Beckman, rotor JA20, 10,000 rpm).
5. Resuspend in 9 ml of H₂O plus 1 ml 5 M NaCl.
6. Centrifuge at 17,000 g for 12 min at 4°C (Beckman, rotor JA20, 12,000 rpm).
7. Resuspend in H₂O.
8. Make a sucrose gradient from 63% to 40% in TE buffer (10mM Tris, 1 mM EDTA, pH 8.0), using a gradient former (MBA, Clearwater, FL) and Masterflex pump (Cole-Parmer Instrument, Co.).
9. Centrifuge in an ultracentrifuge at 100,000 g for 30 min at 4°C (Sorval, rotor AH626, 24,000 rpm).
10. Take the virus band; add H₂O and mix well.
11. Centrifuge at 17,000 g for 12 min at 4°C (Beckman, rotor JA20, 12,000 rpm).
12. Resuspend in H₂O.

APPENDIX B

PURIFICATION OF THE ALKALI RELEASED VIRUS

(Routine protocol in Dr. Maruniak's laboratory).

1. Add 1/3 of the volume of dilute alkaline solution-DAS (final concentration: 0.1 M Na_2CO_3 , 0.01 M EDTA, 0.17 M NaCl, pH 10.9) to the solution containing polyhedra. Keep the polyhedra solution on ice. Observe under the microscope, if polyhedra are not dissolved add from 100 to 500 μl of 0.5 M NaOH and vortex until dissolved.
2. Mix by vortexing.
3. Prepare a sucrose gradient from 40 to 56%.
4. Add the virus on top of the gradient.
5. Ultracentrifuge at 100,000 g for 1 hour (Sorval, rotor AH626, 24,000 rpm).
6. Save the upper phase which contains the polyhedrin.
7. Transfer the region of the different bands, which corresponds to the virion with different number of nucleocapsids, to a new tube.
8. Add TE buffer to fill the tube and mix well. Balance the tubes and ultracentrifuge at 100,000 g for 30 min.
9. Discard supernatant.
10. Resuspend the virus (alkali-released virus) in 500 μl TE buffer.

APPENDIX C

BACULOVIRUS DNA PURIFICATION

(Routine procedure used in Dr. Maruniak's laboratory).

1. Add 40 μ l of 20 % SDS to the alkali-released virus suspension.
2. Incubate at 37°C for 10 min.
3. Add 10 - 25 μ l Proteinase K (5 mg/ml) and incubate from 6 hours to overnight at 37°C.
4. Extract DNA with 0.75 ml phenol saturated in TE buffer. Invert tubes gently for 3 to 4 min. Centrifuge tubes in microcentrifuge for about 1 min. Remove upper aqueous phase and transfer to a clean microfuge tube. Do this step three times.
5. Extract the aqueous phase three times with ether saturated in H₂O. Invert tubes gently for 3 to 4 min. Centrifuge tubes in microcentrifuge for about 1 min. Discard upper phase and keep lower phase.
6. Heat the DNA solution at 56°C for at least 10 min in a heat block with caps open to evaporate ether.
7. Dialyze the DNA solution for two days against 1 liter TE buffer. Change three times daily.
8. Measure the DNA concentration by reading optical density at 260 nm. $OD_{260} \times 50 \times$ dilution factor = micrograms of DNA per ml.

APPENDIX D POLYHEDRIN PCR PROTOCOL

Component	Addition Order	Volume (μl)	Final Concentration
SD. H ₂ O	1	17.75	-
[10x] Reaction Buffer	2	2.5	1x
dNTP's (10 mM each)	3	0.5	200 μM/ nucleotide
50 mM MgCl ₂	4	0.5	1 mM
Primer JM33	5	1.25	12.5 pmoles/reaction
Primer JM34	5	1.25	12.5 pmoles/reaction
PrimeZyme polymerase	6	0.25	0.5 U/reaction
DNA Template	7	1.0	1.0 ng
Final Volume	-	25.0	-

A master mix of reagents for all samples (water, buffer, dNTP's, primers, and enzyme) was prepared first, then aliquoted to the individual tubes (24 μl). After that, the DNA template (1 μl) was added.

At the end 20-25 μl of mineral oil was added to each tube to prevent evaporation during cycling.

APPENDIX E
VIRAL DNA EXTRACTION PROTOCOL FOR INDIVIDUAL LARVAE AND
INDIVIDUAL PREDATORS

(Protocol developed by Rejane R. de Moraes).

1. Place individual insects in microfuge tubes.
2. Add 250 (larva < 1.5 cm) or 500 μ l (larva > 1.5 cm) of homogenization buffer.
3. Grind insect in a Sted Fast stirrer Model SL 300 (Fisher Scientific, Pittsburgh, PA).
4. Centrifuge at 3,000 g for 2 minutes.
5. Resuspend pellet in 500 μ l of 1 M Tris pH 8.0.
6. Centrifuge at 10, 333 g for 10 minutes.
7. Resuspend pellet in 500 μ l of 1 M Tris pH 8.0.
8. Add 1/3 volume of Dilute Alkaline Solution (DAS) and vortex.
9. Use microscope to determine whether the polyhedra have solubilized or not.
10. If polyhedra do not solubilize, add 200-500 μ l of 0.5 M NaOH and vortex.
11. Centrifuge at 10, 333 g for 10 minutes;
12. Resuspend pellet in 200 μ l of 1 M Tris-HCl pH 8.0;
13. Dilute 100 X; Samples are ready for PCR.

APPENDIX F
POLYHEDRA EXTRACTION FROM SOIL AND VIRAL DNA PURIFICATION BY
PHENOL-ETHER EXTRACTIONS

(Protocol developed by Rejane R. de Moraes).

1. A) Add 2 μ l AgMNPV stock solution (5.0×10^9 PIB/ml) in 48 μ l of SD H₂O and inoculate in 0.25 g soil (detection limit experiments).
B) If soil samples came from field, weigh 0.5 g of soil and proceed with step 4.
2. Shake for 10 min at room temperature.
3. Let adsorb for 10 min.
4. Add 1.0 ml 0.1 M Na₄P₂O₇ pH 7.0 and incubate in shaker, at room temperature for 4 hours.
5. Centrifuge at 12,127 g for 10 min.
6. Resuspend pellet by adding 1.0 ml 1 x TE buffer pH 8.0.
7. Centrifuge at 12,127 x g for 10 min.
8. Resuspend pellet with 500 μ l TE buffer pH 8.0, 200 μ l DAS (0.3 M Na₂CO₃, 0.03 M EDTA, 0.51 M NaCl), and 500 μ l 0.2 M NaOH.
9. Incubate on shaker at room temperature for 2 hours.
10. Centrifuge at 1,794 g for 5 min.
11. Save supernatant.
12. Centrifuge at 12,127 g for 20 min at 4°C.
13. Resuspend pellet in 500 μ l TE buffer pH 8.0. Add 20 μ l proteinase K (5 mg/ml) and incubate overnight at 37°C or for 1 h at 60-68°C.
14. Phenol extract 3 times (500 μ l).
15. Ether extract 3 times (500 μ l).

16. Evaporate ether with microfuge tubes caps open at 56°C for 10 min.
17. Dilute 10 or 100 times; samples are ready for PCR amplification.

APPENDIX G
MAGNETIC CAPTURE-HYBRIDIZATION (MCH)

(Modified from Jacobssen, 1996).

1. Wash 200 μ l of streptavidin coated Dynal-beads M-280 (Dynal), for three times, with 400 μ l 1 x PBS, 0.1% SDS pH 7.1.
2. Wash beads once with 400 μ l TE, 1 M NaCl.
3. Resuspend in 200 μ l TE, 1 M NaCl (20 μ l per reaction).
4. Add approximately 100 ng of biotin labeled probe. Incubate at room temperature for 60 min, in a hybridization oven (Biometra, Inc.).
5. Wash three times with 400 μ l TE, 1 M NaCl.
6. Resuspend in 400 μ l of 0.125 M NaOH, 0.1 M NaCl and incubate for 15 min at room temperature in the hybridization oven.
7. Wash three times with 400 μ l TE, 1 M NaCl to remove any NaOH and complementary DNA left.
8. Resuspend in 200 μ l SD H₂O and use for hybridization.
9. To 50 μ l of DNA sample add 330 μ l of hybridization solution (5 x SSC, 0.02% SDS) and 20 μ l of magnetic probe.
10. Hybridize for two hours at 62°C in a hybridization oven.
11. After hybridization, wash beads with 400 μ l SD H₂O, concentrate using magnetic rack and remove supernatant.
12. Wash and resuspend in 50 μ l SD H₂O.
13. Add 25 μ l resuspended beads to 25 μ l aliquots of PCR master mix.

APPENDIX H
POLYHEDRA EXTRACTION FROM SOIL AND VIRAL DNA PURIFICATION TO
BE USED IN THE MCH PROCEDURE

(Procedure developed by Rejane R. de Moraes)

1. A) Add 2 μ l AgMNPV stock solution (5.0×10^9 PIB/ml) to 48 μ l of SD H₂O and inoculate in 0.25 g soil (detection limit experiments).
B) If soil samples came from the field, weigh 0.5 g of soil and proceed with step 4 using twice the volumes indicated on that step.
2. Shake for 10 min at room temperature.
3. Let adsorb for 10 min.
4. Add 300 μ l of 1 x TE buffer pH 8.0 and vortex; add 100 μ l DAS (vortex); add 200 μ l 0.2 NaOH and vortex.
5. Incubate in shaker for one hour at room temperature.
6. Centrifuge at 3,000 g for 5 min and save supernatant.
7. Centrifuge at 20,000 g for 20 min at 4°C.
8. Resuspend pellet in 200 μ l 1 x phosphate buffer saline (PBS).
9. Boil for 10 min.
10. Centrifuge for 10 min at 20,000 g at 4°C. Save supernatant and use 50 μ l of it for the MCH hybridization (Step 9 of the MCH protocol).

APPENDIX I
POLYHEDRIN PCR PROTOCOL FOR AGMNPV DNA FROM SOIL SAMPLES

Component	Addition Order	Volume (μ l)	Final Concentration
SD H ₂ O	1	12.5	-
[10x] Reaction Buffer	2	5.0	1x
dNTP's (10 mM each)	3	1.0	200 μ M/ nucleotide
50 mM MgCl ₂	4	1.0	1 mM
Primer JM33	5	2.5	12.5 pmoles/reaction
Primer JM34	5	2.5	12.5 pmoles/reaction
PrimeZyme polymerase	6	0.5	0.5 U/reaction
DNA Template (isolated by the MCH procedure)	7	25.0	unknown
Final Volume	-	50.0	-

A master mix of reagents for all samples (water, buffer, dNTP's, primers, and enzyme) was prepared first, then aliquoted to the individual tubes (25 μ l). Subsequently, 25 μ l of AgMNPV DNA isolated from soil samples by the MCH procedure was added. At the end 50 μ l of mineral oil was added to each tube to prevent evaporation during cycling.

APPENDIX J COMPETITIVE POLYHEDRIN PCR PROTOCOL

Component	Addition Order	Volume (μl)	Final Concentration
SD H ₂ O	1	5.25	-
[10x] Reaction Buffer	2	2.5	1x
dNTP's (10 mM each)	3	0.5	200 μM/ nucleotide
50 mM MgCl ₂	4	0.5	1 mM
Primer JM33	5	1.25	12.5 pmoles/reaction
Primer JM34	5	1.25	12.5 pmoles/reaction
PrimeZyme polymerase	6	0.25	0.5 U/reaction
AgMNPV Target DNA	7	12.5	unknown
Competitive DNA	8	1.0	0.1 pg
Final Volume	-	25.0	-

A master mix of reagents for all samples (water, buffer, dNTP's, primers, and enzyme) was prepared first, then aliquoted to the individual tubes (11.5 μl). Subsequently, 12.5 μl of AgMNPV target DNA isolated from soil by the MCH procedure was added and followed by the addition of 1.0 μl of pGEM-T₅₁₀ competitor DNA. At the end 20-25 μl of mineral oil was added to each tube to prevent evaporation during cycling.

APPENDIX K ALKALINE BLOTTING

(According to Bio Rad's recommendations)

1. Cut four sheets of Whatman 3MM paper so that they overhang the bottom of the gel tray by approximately 5 cm on each end.
2. Place the four sheets of Whatman 3MM paper on an inverted gel casting tray and place them in the bottom of a deep dish. Saturate the 3MM paper with 0.4 M NaOH. Remove bubbles by repeatedly rolling a glass pipette over the 3MM paper. Pour enough NaOH into the dish so that the ends of the paper are immersed in NaOH.
3. Place the gel containing the separated DNA fragments on the Whatman 3MM paper. Remove bubbles from beneath the gel and cover the gel with a small amount of NaOH.
4. Place Fisher plastic wrap over the entire gel/3MM stack. Cut out a window with a razor blade, allowing only the gel to emerge.
5. Place the pre-wetted sheet of Zeta-Probe membrane onto the gel. Make contact first in the center, then allow the edges of the membrane to gradually fold down. Carefully, add NaOH to the membrane's surface, and remove bubbles.
6. Cut two pieces of 3MM exactly to the gel size. Wet the sheets of pre-cut papers in water and place them, one by one, on the Zeta-Probe membrane/gel stack. Remove bubbles from underneath each paper.
7. Place a stack of pre-cut paper towels on the 3MM/Zeta-Probe membrane/gel stack. Cover the paper towel stack with a glass plate and a small bottle containing water to

make pressure. In this way, the paper will absorb the NaOH buffer in the tray and the DNA will be transferred from the gel to the membrane.

8. Allow transfer for 4-24 hours, depending on the gel concentration and DNA fragment size.
9. After transfer, remove the stack of paper towels, peel the Zeta-Probe membrane gently from the gel surface, rinse it in 2 x SSC (20 x Stock = 175 g NaCl, 88.2 g Na citrate in 1 liter water), and air dry. The dried membranes are stable at room temperature for two days. If hybridization is not to be undertaken in this period of time, the membranes should be vacuum dried at 80°C for 30 min. The membranes can be stored dry between two pieces of filter paper in plastic bags at 4°C.

APPENDIX L
DNA LABELING BY THE NICK TRANSLATION WITH MINIMAL DNASE
METHOD

(Modified from the USB Nick Translation Kit Protocol)

1. For a total reaction volume of 50 μ l, add the following to a microfuge tube:
 - 30 μ l of DNA (0.2 μ g)
 - 5 μ l 10 x NT buffer
 - 2 μ l of 0.4 mM dATP in 10 mM Tris pH 7.7
 - 2 μ l of 0.4 mM dGTP in 10 mM Tris pH 7.7
 - 2 μ l of 0.4 mM dTTP in 10 mM Tris pH 7.7
 - 1 μ l DNase I (1000 fold diluted from a 0.005 U/ μ l stock, in 10 mM Tris pH 7.7)
 - 2 μ l DNA polymerase I (holoenzyme from *Escherichia coli*)
 - 30 μ Ci of ^{32} P-dCTP
 - Sterile-distilled H₂O to complete 50 μ l
2. Incubate at 15°C for 15 min.
3. Heat-inactivate enzymes at 65°C for 10 min. Keep on ice.
4. To remove unincorporated nucleotides, precipitate labeled DNA with ethanol three times as described below:
5. Add 1 μ l tRNA (1 mg/ml), to serve as a carrier.
6. Add 25 μ l of 7.5 M ammonium acetate (1/2 volume).
7. Add 150 μ l absolute ethanol (2 x volume), and mix.
8. Incubate on ice for 10 min.
9. Centrifuge in microcentrifuge for 10 min. Remove supernatant and resuspend pellet in 50 μ l 1 x TE buffer. Check pellet with Geiger counter to make sure the radioactive

DNA was not lost. Repeat items 6-9 twice. At the last time, dry DNA pellet in vacuum for 10 min and then resuspend pellet in 50 μ l 1 x TE buffer.

10. Add 100 μ l 0.2 N NaOH to denature probe and boil for 5 min.

11. Quench on ice.

12. Add 100 μ l 1 M Tris pH 8.0 to neutralize NaOH.

13. Place probe in the hybridization solution and proceed with hybridization.

LIST OF REFERENCES

- Abbas, M. S. T. & D. G. Boucias. 1984. Interaction between nuclear polyhedrosis virus-infected *Anticarsia gemmatilis* (Lepidoptera:Noctuidae) larvae and predator *Podisus maculiventris* (Say) (Hemiptera:Pentatomidae). *Env. Entomol.* 13: 599-602.
- Abbaszadegan, M., M. S. Huber, C. P. Gerba & I. L. Pepper. 1993. Detection of enteroviruses in groundwater with the polymerase chain reaction. *Appl. Environ. Microbiol.* 59: 1318-1324.
- Adams, J. R., R. H. Goodwin & T. A. Wilcox. 1977. Electron microscopic investigations on invasion and replication of insect baculovirus in vivo and in vitro. *Review of Biology and Cell.* 28: 261-268.
- Agresti, A. 1990. Describing two-way contingency tables, pp. 30. In: *Categorical Data Analysis*. A. Agresti, Eds. John Wiley & Sons, Inc, New York, NY.
- Allen, G. E. & J. D. Knell. 1977. A nuclear polyhedrosis virus of *Anticarsia gemmatilis*. I. Ultrastructure, replication and pathogenicity. *Florida Entomologist* 60, 233-240.
- Ansari, S. A., S. R. Farrah & G. R. Chadhry. 1992. Presence of human immunodeficiency virus nucleic acids in wastewater and their detection by Polymerase Chain Reaction. *Appl. Environ. Microbiol.* 58: 3984-3990.
- Arif, B. M. & W. Doerfler. 1984. Identification and localization of reiterated sequences in the *Choristoneura fumiferana* MNPV genome. *The EMBO Journal* 3: 525-529.
- Ayres, M. D., S. C. Howard, J. Kuzio, M. Lopez-Ferber & R. D. Possee. 1994. The complete sequence of *Autographa californica* nuclear polyhedrosis virus. *Virology* 202: 586-605.
- Beames, B. & M. D. Summers. 1988. Comparisons of host cell DNA insertions and altered transcription at the start site of insertions in few polyhedra baculovirus mutants. *Virology* 162: 206-220.

- Beames, B. & M. D. Summers. 1989. Location and nucleotide sequence of the 25K protein missing from baculovirus few polyhedra (FP) mutants. *Virology* 168: 344-353.
- Beekman, A. G. 1980. The infectivity of polyhedra of nuclear polyhedrosis virus (N.P.V.) after passage through gut of an insect-predator. *Experientia* 36: 858-859.
- Benz., G. A. 1986. Introduction: Historical Perspectives. *In: The Biology of Baculovirus: Biological Properties and Molecular Biology*. Granados, R. R., and B. A. Federici, Eds. CRC Press, Inc., Boca Raton, Florida. Vol. 1, pp. 1-36.
- Berthelet, M., L.G. Whyte & C.W. Greer. 1996. Rapid, direct extraction of DNA from soils for PCR analysis using polyvinylpyrrolidone spin columns. *FEMS Microbiology Letters* 138: 17-22.
- Bird, F. T. & M. M. Whalen. 1953. A virus disease of the European pine sawfly, *Neodiprion sertifer* (Geoffr.). *Can. Entomol.* 85: 433-437.
- Bischoff, D. S. & J. M. Slavicek. 1996. Characterization of the *Lymantria dispar* nucleopolyhedrovirus 25K FP gene. *J. Gen. Virol.* 77: 1913-1923.
- Bishop, D. H. L. 1986. UK release of genetically marked virus. *Nature* 323: 496.
- Bishop, D. H. L. 1989. Genetically-engineered viral insecticides-a progress report 1986-1989. *Pestic. Sci.* 27: 173-189.
- Bitton, G., M.J. Charles & S.R. Farrah. 1979. Virus detection in soils: a comparison of four recovery methods. *Can J. Microbiol.* 25: 874-880.
- Bonning, B. C. & B. D. Hammock. 1992. Development and potential of genetically engineered viral insecticides. *Biotech. Eng. Rev.* 10: 455-489.
- Bonning, B. C., M. Hirst, R. D. Possee & B. D. Hammock. 1992. Further development of a recombinant baculovirus insecticide expressing the enzyme juvenile hormone esterase from *Heliothis virescens*. *Insect Biochem. Mol. Biol.* 22: 453-458.
- Bonning, B. C., P. W. Roelvink, J. M. Vlak, R. D. Possee & B. D. Hammock. 1994. Superior expression of juvenile hormone esterase and β -galactosidase from the basic protein promoter of *Autographa californica* nuclear polyhedrosis virus compared to the p10 protein and polyhedrin promoters. *J. Gen. Virol.* 75: 1551-1556.

- Borchers, K. & J. Slater. 1993. A nested PCR for the detection and differentiation of EHV-1 and EHV-4. *J. Virol. Methods* 45: 331-336.
- Boucias, D.G., M.S.T. Abbas, L. Rathbone & N. Hostettler. 1987. Predators as potential dispersal agents of the nuclear polyhedrosis virus of *Anticarsia gemmatilis* (Lep.: Noctuidae) in soybean. *Entomophaga* 32: 97-108.
- Braunagel, S. C. & M. D. Summers. 1994. *Autographa californica* nuclear polyhedrosis virus, PDV, and ECV viral envelopes and nucleocapsids: structural proteins, antigens, lipid and fatty acid profiles. *Virology* 202: 315-328.
- Brown, D. A., C. J. Allen & G. N. Bignell. 1982. The use of a protein A conjugate in an indirect enzyme-linked immunosorbent assay (ELISA) of four closely related baculoviruses from *Spodoptera* species. *J. Gen. Virol.* 62: 375-378.
- Burand, J. P., H. M. Horton, S. Retnasami & J. S. Elkington. 1992. The use of polymerase chain reaction and shortwave UV irradiation to detect baculovirus DNA on the surface of gypsy moth eggs. *J. Virol. Methods* 36: 141-150.
- Capinera, J. L. & P. Barbosa. 1975. Transmission of nuclear-polyhedrosis virus to gypsy moth larvae by *Calosoma sycophanta*. *Ann. Entomol. Soc. Am.* 68: 593-594.
- Carbonell, L. F., M. R. Hodge, M. D. Tomalski & L. K. Miller. 1988. Synthesis of a gene coding for an insect-specific scorpion neurotoxin and attempts to express it using baculovirus vectors. *Gene* 73: 409-418.
- Carbonell, L. F. & L. K. Miller. 1987. Baculovirus interaction with nontarget organisms: a virus-borne reporter gene is not expressed in two mammalian cell lines. *Appl. Environ. Microbiol.* 7: 1412-1417.
- Carner, G. R. & S. G. Turnipseed. 1977. Potential of the nuclear polyhedrosis virus of the velvetbean caterpillar for eight noctuid hosts. *J. Invertebr. Pathol.* 33: 211-216.
- Carson, D. D., M. D. Summers & L. A. Guarino. 1991. Transient Expression of the *Autographa californica* nuclear polyhedrosis virus immediate-early gene, IE-N, is regulated by three viral elements. *J. Virol.* 65: 945-951.
- Chang, M. T., C. Lanner-Herrera & M. Fikes. 1989. Nucleotide sequence of *Lymantria dispar* nuclear polyhedrosis virus polyhedrin gene. *J. Invertebr. Pathol.* 53: 241-246.
- Chisholm, G. E. & D. J. Henner. 1988. Multiple early transcripts and splicing of the *Autographa californica* nuclear polyhedrosis virus IE-1 gene. *J. Virol.* 62: 3193-3200.

- Cochran, M. A., E. B. Carstens, B. T. Eaton & P. Faulkner. 1982. Molecular cloning and physical mapping of restriction endonuclease fragments of *Autographa californica* nuclear polyhedrosis virus DNA. *J. Virol.* 41: 940-946.
- Cochran, M. A. & P. Faulkner. 1983. Location of homologous DNA sequences interspersed at five regions in the Baculovirus AcMNPV genome. *J. Virol.* 45:961-970.
- Cooper, D. J. 1981. The role of predatory Hemiptera in disseminating a nuclear polyhedrosis virus of *Heliothis punctigera*. *J. Aust. Entomol. Soc.* 20: 145-150.
- Cory, J. S., M. L. Hirst, T. Williams, R. S. Hails, D. Goulson, B. M. Green, T. M. Carty, R. D. Possee, P. J. Cayley & D. H. L. Bishop. 1994. Field trial of a genetically improved baculovirus insecticide. *Nature* 370: 138-140.
- Couch, J. A. 1974. An enzootic nuclear polyhedrosis virus of pink shrimp: ultrastructure, prevalence, and enhancement. *J. Invertebr. Pathol.* 24: 311-331.
- Cowan, P., D. Bulach, K. Goodge, A. Robertson & D. E. Tribe. 1994. Nucleotide sequence of the polyhedrin gene region of *Helicoverpa zea* single nucleocapsid nuclear polyhedrosis virus: placement of the virus in lepidopteran nuclear polyhedrosis virus group II. *J. Gen. Virol.* 75: 3211-3218.
- Crawford, A. M., P. Faulkner & J. Kalmakoff. 1978. Comparison of solid-phase radioimmunoassays for baculoviruses. *Appl. Environ. Microbiol.* 36: 18-24.
- Crawford, A. M., J. Kalmakoff & J. F. Longworth. 1977. A solid-phase radioimmunoassay for polyhedron protein from baculoviruses. *Interviol.* 8: 117-121.
- Crawford, A. M. & L. K. Miller. 1988. Characterization of an early gene accelerating expression of late genes of the baculovirus *Autographa californica* nuclear polyhedrosis virus. *J. Virol.* 62: 2773-2781.
- Crook, N. E. & C. C. Payne. 1980. Comparison of three methods of ELISA for baculoviruses. *J. Gen. Virol.* 46: 29-37.
- Crowe, J. S., H. J. Cooper, M. A. Smith, M. J. Sims, D. Parker & D. Gewert. 1991. Improved cloning efficiency of polymerase chain reaction (PCR) products after proteinase K digestion. *Nucleic Acid Research* 19: 184.
- David, W. A. L., & B. O. Gardiner. 1966. Persistence of a granulosis virus of *Pieris brassicae* on cabbage leaves. *J. Invertebr. Pathol.* 8: 180-183.

- David, W. A. L., & B. O. Gardiner. 1967a. The effect of heat, cold, and prolonged storage on a granulosis virus of *Pieris brassicae*. *J. Invertebr. Pathol.* 9: 555-562.
- David, W. A. L., & B. O. Gardiner. 1967b. The persistence of a granulosis virus of *Pieris brassicae* in soil and sand. *J. Invertebr. Pathol.* 9: 342-347.
- David, W. A. L., B. O. Gardiner & M. Woolner. 1968. The effects of sunlight on a purified granulosis virus of *Pieris brassicae* applied to cabbage leaves. *J. Invertebr. Pathol.* 11: 496-501.
- Devereaux, J., P. Haeberli & O. Smithies. 1984. A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res.* 12: 387-396.
- Eldridge, R., F. M. Horodyski, D. B. Morton, D. R. O'Reilly, J. W. Truman, L. M. Riddiford & L. K. Miller. 1991. Expression of an eclosion hormone gene in insect cells using baculovirus vectors. *Insect Biochem.* 21: 341-351.
- Eldridge, R., D. R. O'Reilly, B. D. Hammock & L. K. Miller. 1992. Insecticidal properties of genetically engineered baculoviruses expressing an insect juvenile hormone esterase gene. *Appl. Environ. Microbiol.* 58: 1583-1591.
- Entwistle, P. F. & P. H. W. Adams. 1977. Prolonged retention of infectivity in the nuclear polyhedrosis virus of *Gilpinia hercyniae* (Hymenoptera, Diprionidae) on foliage of spruce species. *J. Invertebr. Pathol.* 29: 392-394.
- Entwistle, P. F. & H. F. Evans. 1985. Viral Control. *In: Comprehensive Insect Physiology, Biochemistry and Pharmacology.* L. I. Gilbert & G. A. Kerkut, Eds. Volume 12. Pergamon Press, Oxford. pp. 347-412.
- Evans, H. F. 1986. Ecology and Epizootiology of Baculoviruses. *In: The Biology of Baculoviruses: Biological Properties & Molecular Biology.* R. R. Granados & B. A. Federici, Eds. Vol. 2, CRC Press, Boca Raton, Florida. pp. 89-132.
- Evans, H. F. & P. F. Entwistle. 1987. Viral Diseases. *In: Epizootiology of Insect Diseases.* J. R. Fuxa & Y. Tanada, Eds. John Wiley & Sons, New York. pp. 257-322.
- Evans, H. F. & K. A. Harrap. 1982. Persistence of insect viruses. *In: Virus Persistence.* B. W. J. Mahy, A. C. Minson & G. Darby, Eds. 33rd Symposium of Soc. Microbiol. Cambridge University Press, London. pp. 57-98.
- Falk, K., J. W. Gratama, M. Rowe, J. Z. Zou, F. Khanim, L. S. Young, M. A. P. Oosterveer & I. Ernberg. 1995. The role of repetitive DNA sequences in the size variation of Epstein-Barr virus (EBV) nuclear antigens, and the

- identification of different EBV isolates using RFLP and PCR analysis. *J. Gen. Virol.* 76: 779-790.
- Farrah, S.R., P.R. Scheuerman & G. Bitton. 1981. Urea-Lysine method for recovery of enteroviruses from sludge. *Appl. Environ. Microbiol.* 41: 455-458.
- Federici, B. A. 1986. Ultrastructure of baculoviruses. *In: Biology of Baculoviruses: Biological Properties and Molecular Biology.* Granados, R. R. & B. A. Federici, Eds. Vol. 1, CRC Press, Inc., Boca Raton, Florida. pp.61-88.
- Ferre, F. 1992. Quantitative or semi-quantitative PCR: reality versus myth. *PCR Methods Appl.* 2: 1-9.
- Ferre, F., A. Marchese, P. C. Duffy, D. E. Lewis, M. R. Wallace, H.J. Beecham, K. G. Burnett, F. C. Jensen & D. J. Carlo. 1992. Quantitation of HIV viral burden by PCR in HIV seropositive navy personnel representing Walter Reed stages 1 to 6. *AIDS Res. Hum. Retrovir.* 8: 269-275.
- Fiksel, J. R. & V. T. Covello. 1986. The suitability and applicability of risk assessment methods and environmental applications of biotechnology. *In: Biotechnology risk assessment. Issues and methods for environmental introduction.* J. Fiksel & V. T. Covello, Eds. pp. 1-34
- Fraser, M. J. & W. F. Hink. 1982. The isolation and characterization of the MP and FP plaque variants of *Galleria mellonella* polyhedrosis virus. *Virology* 117: 366-378.
- Friesen, P. D. & L. K. Miller. 1986. The regulation of baculovirus gene expression. *Current Topics of Microbiol. and Immunol.* 131: 31-49.
- Fuchs, Y. L., M. S. Woods & R. F. Weaver. 1983. Viral transcription during *Autographa californica* nuclear polyhedrosis virus infection: a novel RNA polymerase induced in infected *Spodoptera frugiperda* cells. *J. Virol.* 48: 641-646.
- Funderburk, J. E. & T. P. Mack. 1987. Abundance and dispersion of *Geocoris* spp. (Hemiptera:Lygaeidae) in Alabama and Florida soybean fields. *Florida Entomol.* 70: 432-439.
- Funderburk, J. E. & T. P. Mack. 1989. Seasonal abundance and dispersion patterns of damsel bugs (Hemiptera:Nabidae) in Alabama and Florida soybean fields. *J. Entomol. Sci.* 24: 9-15.
- Funderburk, J., J.E. Maruniak, D. Boucias, & A. Garcia-Canedo. 1992. Efficacy of baculoviruses and their impact on pest management programs. *In: Pest*

Management in Soybean. L.G. Copping, M. Green & R. Rees, Eds. Elsevier, England. pp. 88-97.

- Fuxa, J.R. & A.R. Richter. 1993. Lack of vertical transmission in *Anticarsia gemmatilis* (Lepidoptera:Noctuidae) nuclear polyhedrosis virus, a pathogen not indigenous to Louisiana. Environ. Entomol. 22: 425-431.
- Fuxa, J.R. & A.R. Richter. 1994. Distance and rate of spread of *Anticarsia gemmatilis* (Lepidoptera: Noctuidae) nuclear polyhedrosis virus released into soybean. Environ. Entomol. 23: 1308-1316.
- Fuxa, J.R., A.R. Richter & M.S. Strother. 1993. Detection of *Anticarsia gemmatilis* nuclear polyhedrosis virus in predatory arthropods and parasitoids after viral release in Louisiana soybean. J. Entomol. Sci. 28: 51-60.
- Fuxa, J. R. & Y. Tanada. 1987. Epidemiological concepts applied to insect epizootiology. In: Epizootiology of Insect Diseases. J. R. Fuxa & Y. Tanada, Eds. John Wiley & Sons, New York. pp. 3-22.
- Fuxa, J.R., G.W. Warren, & C. Kawanish. 1985. Comparisom of bioassay and Enzyme-Linked Immunosorbent assay for quantification of *Spodoptera frugiperda* Nuclear Polyhedrosis Virus in soil. J. Invertebr. Pathol. 46: 133-138.
- Garcia-Maruniak, A., O. H. O. Pavan & J. E. Maruniak. 1996. A variable region of *Anticarsia gemmatilis* nuclear polyhedrosis virus contains tandemly repeated DNA sequences. Virus Research 41: 123-132.
- Gonzalez, M. A., G. E. Smith & M. D. Summers. 1989. Insertion of the SfMNPV polyhedrin gene into an AcMNPV polyhedrin deletion mutant during viral infection. Virology 170: 160-175.
- Granados, R. R. 1978. Early events in the infection of *Heliothis zea* midgut cells by a baculovirus. Virology 90:170-174.
- Granados, R. R. 1980. Infectivity and mode of action of baculoviruses. Biotechnol. Bioeng. 22: 65-68.
- Granados, R. R. & K. A. Lawler. 1981. In vivo pathway of *Autographa californica* baculovirus invasion and infection. Virology 108: 297-308.
- Granados, R. R. , K. A. Lawler & J. P. Burand. 1981. Replication of *Heliothis zea* baculovirus in an insect cell line. Interviol. 16: 71-79.

- Grula, M. A., P. L. Buller & R. F. Weaver. 1981. Alpha amanitin-resistant viral RNA synthesis in nuclei isolated from nuclear polyhedrosis virus-infected *Heliothis zea* larvae and *Spodoptera frugiperda* cells. *J. Virol.* 38: 916-921.
- Guarino, L. A. & W. Dong. 1991. Expression of an enhancer-binding protein in insect cells transfected with the *Autographa californica* nuclear polyhedrosis virus IE1 gene. *J. Virol.* 65: 3676-3680.
- Guarino, L. A., M. A. Gonzalez & M. D. Summers. 1986. Complete sequence and enhancer function of the homologous DNA regions of *Autographa californica* nuclear polyhedrosis virus. *J. Virol.* 60: 224-229.
- Guarino, L. A. & M. D. Summers. 1986a. Functional mapping of a trans-activating gene required for expression of a baculovirus delayed-early gene. *J. Virol.* 57: 563-571.
- Guarino, L. A. & M. D. Summers. 1986b. Interspersed homologous DNA of *Autographa californica* nuclear polyhedrosis virus enhances delayed-early gene expression. *J. Virol.* 60: 215-223.
- Gudauskas, R. T. & D. Canerday. 1968. The effect of heat, buffer, salt and H-ion concentration, and ultraviolet light on the infectivity of *Heliothis* and *Trichoplusia* nuclear-polyhedrosis viruses. *J. Invertebr. Pathol.* 12: 405-411.
- Hammock, B. D., B. C. Bonning, R. D. Possee, T. N. Hanzlik & S. Maeda. 1990. Expression and effects of the juvenile hormone esterase in a baculovirus vector. *Nature* 344: 458-461.
- Harlow, S. P. & C. C. Stewart. 1993. Quantitation of c-myc gene amplification by a competitive PCR assay system. *PCR Methods Appl.* 3: 163-168.
- Harrap, K. A. 1972. The structure of nuclear polyhedrosis viruses. I. The inclusion body. *Virology* 50: 114-123.
- Harrison, R. L. & M. D. Summers. 1995. Mutations in the *Autographa californica* nuclear polyhedrosis virus 25 Kda protein gene result in reduced virion occlusion, altered intranuclear envelopment and enhanced virus production. *J. Gen. Virol.* 76: 1451-1459.
- Hayward-Lester, A., P. J. Oefner & P. A. Doris. 1996. Rapid quantification of gene expression by competitive RT-PCR and ion-pair reversed-phase HPLC. *Biotechniques* 20: 250-257.
- Hillier, L. & P. Green. 1991. OSP: A computer program for choosing PCR and DNA sequencing primers. *PCR Methods and Applications* 1: 124-128.

- Hooft Van Iddekinge, B. J. L., G. E. Smith & M. D. Summers. 1983. Nucleotide sequence of the polyhedrin gene of *Autographa californica* nuclear polyhedrosis virus. *Virology* 131: 561-565.
- Hostetter, D. L. 1971. A virulent nuclear polyhedrosis virus of the cabbage looper, *Trichoplusia ni*, recovered from the abdomens of sarcophagid flies. *J. Invertebr. Pathol.* 17: 130-131.
- Huber, J. 1982. The baculoviruses of *Cydia pomonella* and other tortricids. *In: Proc. IIIrd Int. Colloq. Invertebr. Pathol.* Brighton. pp. 119.
- Hughes, K. M. & R. B. Addison. 1970. Two nuclear polyhedrosis viruses of the Douglas-fir tussock moth. *J. Invertebr. Pathol.* 16: 196-200.
- Huh, N. E. & R. F. Weaver. 1990. Identifying the RNA polymerases that synthesize specific transcripts of the *Autographa californica* nuclear polyhedrosis virus. *J. Gen. Virol.* 71: 195-201.
- Hukuhara, T. 1975. Purification of a cytoplasmic polyhedrosis virus from soil. *J. Invertebr. Pathol.* 25: 337-342.
- Hukuhara, T. & H. Wada. 1972. Adsorption of polyhedra of a cytoplasmic-polyhedrosis virus by soil particles. *J. Invertebr. Pathol.* 20: 309-316.
- Iatrou, K., K. Ito & H. J. Witkiewicz. 1985. Polyhedrin gene of *Bombyx mori* nuclear polyhedrosis virus. *J. Virol.* 54: 436-445.
- Ignoffo, C. M. 1968. Specificity of insect viruses. *Bull. Entomol. Soc. Am.* 14: 265-269.
- Ignoffo, C. M. 1973. Development of a viral insecticide: concept to commercialization. *Exp. Parasitol.* 33: 380-385.
- Ignoffo, C. M. & T. L. Couch. 1981. The nucleopolyhedrosis virus of *Heliothis* species as a microbial insecticide. *In: Microbial Control of Pests and Diseases 1970-1980.* Burges, H. D., Ed. Academic Press, New York, pp. 329.
- Jacobsen, C. S. 1995. Microscale detection of specific bacterial DNA in soil with a magnetic capture-hybridization and PCR amplification assay. *Appl. Environ. Microbiol.* 61: 3347-3352.
- Jacobsen, C. S. 1996. Detection of microbial genes from the rhizosphere by magnetic capture hybridization and subsequent amplification of target genes by PCR. *Mol. Microbiol. Ecol. Manual* 1.4.1: 1-8.

- Jacobsen, C.S. & O.F. Rasmussen. 1992. Development and application of a new method to extract bacterial DNA from soil based on separation of bacteria from soil with cation-exchange resin. *Appl. Environ. Microbiol.* 58: 2458-2462.
- Jalava, T., P. Lehtovaara, A. Kallio, M. Ranki & H. Söderlund. 1993. Quantification of hepatitis B virus DNA by competitive amplification and hybridization on microplates. *Biotechniques* 15: 134-139.
- Jaques, R. P. 1967. The persistence of a nuclear polyhedrosis virus in the habitat of the host insect, *Trichoplusia ni*. II. Polyhedra in soil. *Can. Ent.* 99: 820-829.
- Jaques, R. P. 1975. Persistence, accumulation, and denaturation of nuclear polyhedrosis and granulosis viruses. In: *Baculoviruses for insect pest control: safety considerations*. M. Summers, R. Engler, L. A. Falcon & P. Vail, Eds. Washington, DC. American Society for Microbiology. pp 90-101.
- Johnson, D. W. & J. E Maruniak. 1989. Physical map of *Anticarsia gemmatilis* nuclear polyhedrosis virus (AgMNPV-2D) DNA. *J. Gen. Virol.* 70: 1877-1883.
- Kaupp, W. J. & R. F. Burke. 1984. A staining technique for the inclusion bodies of a granulosis virus. *Forestry Canada FPMI Tech. Note* 1, 2 pp.
- Kaupp, W. J. & P. M. Ebling. 1993. Horseradish peroxidase-labelled probes and enhanced chemiluminescence to detect baculoviruses in gypsy moth and eastern spruce budworm larvae. *J. Virol. Methods* 44: 89-98.
- Keating, S. T., J. P. Burand, & J. S. Elkington. 1989. DNA hybridization assay for detection of gypsy moth nuclear polyhedrosis virus in infected gypsy moth (*Lymantria dispar*L.) larvae. *Appl. Environ. Microbiol.* 55: 2749-2754.
- Keddie, A. B. & L. E. Volkman. 1985. Infectivity difference between the two phenotypes of *Autographa californica* nuclear polyhedrosis virus in an insect host. *Science* 243: 1728-1730.
- Kelly, D. C. 1982. Baculovirus replication. *J. Gen Virol.* 63: 1-13.
- Kelly, D. C. & T. Lescott. 1981. Baculovirus replication: protein synthesis in *Spodoptera frugiperda* cells infected with *Trichoplusia ni* nuclear polyhedrosis virus. *Microbiologica* 4: 35-47.
- King, L. A. & R. D. Possee. 1992. The baculovirus expression System. A laboratory guide. Chapman & Hall. 229 pp.

- Knell, J. D., M. D. Summers and G. E. Smith. 1983. Serological analysis of 17 baculoviruses from subgroups A and B using protein blot immunoassay. *Virology* 125: 381-392.
- Kool, M., R. Broer, D. Zuidema, R. W. Goldbach & J. M. Vlak. 1994. Nucleotide sequence and genetic organization of a 7.3 kb region (map unit 47 to 52.5) of *Autographa californica* nuclear polyhedrosis virus. *J. Gen. Virol.* 75: 487-494.
- Kool, M., R. W. Goldbach & J. M. Vlak. 1994. A putative non-hr origin of DNA replication in the HindIII-K fragment of *Autographa californica* multiple nucleocapsid nuclear polyhedrosis virus. *J. Gen. Virol.* 75: 3345-3352.
- Kool, M., P. M. M. Van Den Berg, J. Tramper, R. W. Goldbach & J. M. Vlak. 1993. Location of two putative origins of DNA replication of *Autographa californica* nuclear polyhedrosis virus. *Virology* 192: 94-101.
- Kukan, B. & J. H. Myers. 1995. DNA hybridization assay for detection of nuclear polyhedrosis virus in tent caterpillars. *J. Invertebr. Pathol.* 66: 231-236.
- Kuzio, J. & P. Faulkner. 1984. Regions of repeated DNA in the genome of *Choristoneura fumiferana* nuclear polyhedrosis virus. *Virology* 139: 185-188.
- Kuzio, J., R. Jaques & P. Faulkner. 1989. Identification of p74, a gene essential for virulence of baculovirus occlusion bodies. *Virology* 173: 759-763.
- Langridge, W. H. R., R. R. Granados & J. F. Greenburg. 1981. Detection of baculovirus protein in cell culture and insect larvae by enzyme-linked immunosorbent assay (ELISA). *J. Gen. Virol.* 54: 443-448.
- Lee, S. -Y., J. Bollinger, D. Bezdicek & A. Ogram. 1996. Estimation of the abundance of an uncultured soil bacterial strain by a competitive quantitative PCR method. *Appl. Environ. Microbiol.* 62: 3787-3793.
- Lees, D. N., K. Henshilwood & W. J. Doré. 1994. Development of a method for detection of enteroviruses in shellfish by PCR with poliovirus as a model. *Appl. Environ. Microbiol.* 60: 2999-3005.
- Leisy, D. J., C. Rasmussen, H. T. Kim & G. F. Rohrmann. 1995. The *Autographa californica* nuclear polyhedrosis virus homologous region 1a: Identical sequences are essential for DNA replication activity and transcriptional enhancer function. *Virology* 208: 742-752.
- Leisy, D. J. & G. F. Rohrmann. 1993. Characterization of the replication of plasmids containing hr sequences in baculovirus-infected *Spodoptera frugiperda* cells. *Virology* 196: 722-730.

- Leisy, D. J., G. F. Rohrmann & G. S. Beaudreau. 1986. The nucleotide sequence of the polyhedrin gene region from the multicapsid nuclear polyhedrosis virus of *Orgyia pseudotsugata*. *Virology* 153: 280-288.
- Leppla, N. C., T. R. Ashley, R. H. Guy & G. D. Butler. 1977. Laboratory life history of the velvetbean caterpillar. *Ann. Entomol. Soc. Am.* 70: 217-220.
- Leser, T. D., M. Boye & N. B. Hendriksen. 1995. Survival and activity of *Pseudomonas* sp. strain B13 (FR1) in a marine microcosm determined by quantitative PCR and an rRNA-targeting probe and its effect on the indigenous bacterioplankton. *Appl. Environ. Microbiol.* 61: 1201-1207.
- Leung, K., L. S. England, M. B. Cassidy, J. T. Trcvors & S. Weir. 1994. Microbial diversity in soil: effect of releasing genetically engineered microorganisms. *Molecular Ecology* 3: 413-422.
- Levin, M. A. Review of environmental risk assessment studies sponsored by EPA. *Recomb. DNA Tech. Bull.* 5: 177-180.
- Lewis, F. B. 1981. Control of the gypsy moth by a baculovirus. *In: Microbial Control of Pests and Diseases, 1970-1980.* Burges, H. D., Ed. Academic Press, New York. pp. 363.
- Li, S., M. Erlandson, D. Moody & C. Gillot. 1997. A physical map of the *Mamestra configurata* nucleopolyhedrovirus genome and sequence analysis of the polyhedrin gene. *J. Gen. Virol.* 78: 265-271.
- Liu, A., J. Qin, C. Rankin, S. E. Hardin & R. F. Weaver. 1986. Nucleotide sequence of a portion of the *Autographa californica* nuclear polyhedrosis virus genome containing the EcoRI site-rich region (hr5) and an open reading frame just 5' of the p10 gene. *J. Gen. Virol.* 67: 2565-2570.
- Liu, J. -C. 1997. Phylogenetic analysis of baculoviruses using gp41 structural protein gene and five other genes. Ph.D. dissertation, University of Florida, Gainesville, FL.
- Liu, J. -C. & J. E. Maruniak. 1995. Nucleotide sequence and transcriptional analysis of the gp41 gene of *Spodoptera frugiperda* nuclear polyhedrosis virus. *J. Gen. Virol.* 76: 1443-1450.
- Longworth, J. F. & G. P. Carey. 1980. The use of an indirect enzyme-linked immunosorbent assay to detect baculovirus in larvae and adults of *Oryctes rhinoceros* from Tonga. *J. Gen. Virol.* 47: 431-438.

- Lu, A. & E. B. Carstens. 1991. Nucleotide sequence of a gene essential for viral DNA replication in the baculovirus *Autographa californica* nuclear polyhedrosis. *Virology* 181: 336-347.
- Luckow, V. A. & Summers, M. D. 1988. Trend in the development of baculovirus expression vectors. *Bio/Technology* 6: 47-55.
- Ma, S. -W., B. G. Corsaro, P. E. Klebba & M. J. Fraser. 1993. Cloning and sequence analysis of a p40 structural protein gene of *Helicoverpa zea* nuclear polyhedrosis virus. *Virology* 192: 224-233.
- Maeda, S. 1989. Increased insecticidal effect by a recombinant baculovirus carrying a synthetic diuretic hormone gene. *Bioche. Biophys. Res. Commun.* 165: 1177-1183.
- Maeda, S., S. L. Volrath, T. N. Hanzlik, S. A. Harper, K. Majima, D.W. Maddox, B. D. Hammock & E. Fowler. 1991. Insecticidal effects of an insect-specific neurotoxin expressed by a recombinant baculovirus. *Virology* 184: 777-780.
- Majima, K., R. Kobara & S. Maeda. 1993. Divergence and evolution of homologous regions of *Bombyx mori* nuclear polyhedrosis virus. *J. Virol.* 67: 7513-7521.
- Maniatis, T., E. F. Fritsch & J. Sambrook. 1989. *Molecular Cloning: A Laboratory Manual*, 2nd Ed. Cold Spring Harbor Laboratory.
- Martens, J. W. M., G. Honne, D. Zuidema, J. W. M. Van Lent and B. Visser. 1990. Insecticidal activity of a bacterial crystal protein expressed by a recombinant baculovirus in insect cells. *App. Environ. Microbiol.* 56: 2764-2770.
- Martens, J. W. M., M. Knoester, F. Weijts, S. J. A. Groffen, Z. Hu, D. Bosch & J. M. Vlak. 1995. Characterization of baculovirus insecticides expressing tailored *Bacillus thuringiensis* CryIA(b) crystal proteins. *J. Invertebr. Pathol.* 66: 249-257.
- Martignoni, M. E. 1978. Virus in biological control: production, activity, and safety. *USDA For. Serv. Tech. Bull.* 1585: 140-145.
- Martignoni, M. E. & P. J. Iwai. 1986. *A catalog of viral diseases of insects, mites, and ticks*, 4th ed. Portland, Oregon: USDA Forest Service PNW-195.
- Martignoni, M. E., P. J. Iwai & G. Rohrmann. 1980. Serum neutralization of nucleopolyhedrosis viruses (baculovirus subgroup A) pathogenic for *Orygia pseudotsugata*. *J. Invertebr. Pathol.* 36: 12-20.

- Maruniak, J. E. 1986. Baculovirus structural proteins and protein synthesis. *In: The Biology of Baculovirus: Biological Properties and Molecular Biology.* Granados, R. R., and B. A. Federici, Eds. CRC Press, Inc., Boca Raton, Florida. Vol. 1, pp. 129-146.
- Maruniak, J. E., S. E. Brown & D. L. Knudson. 1984. Physical maps of SfMNPV baculovirus DNA and its genomic variants. *Virology* 36: 221-234.
- McCarthy, W. J. & L. S. Henchal. 1983. Detection of *Autographa californica* baculovirus nonoccluded virions in vitro and in vivo by enzyme-linked immunosorbent assay. *J. Invertebr. Pathol.* 41: 401-404.
- McCarthy, W. J. & J. T. Lambiase. 1979. Serological relationships among Plusiinae baculoviruses. *J. Invertebr. Pathol.* 34: 170-177.
- McClintock, J. T. & E. M. Dougherty. 1988. Restriction mapping of *Lymantria dispar* nuclear polyhedrosis virus DNA: Localization of the polyhedrin gene and identification of four homologous regions. *J. Gen. Virol.* 69: 2303-2312.
- McCulloch, R. K., C. S. Choong & D. M. Hurley. 1995. An evaluation of competitor type and size for use in the determination of mRNA by competitive PCR. *PCR Methods Appl.* 4: 219-226.
- McCutchen, B. F., P. V. Choudary, R. Crenshaw, D. Maddox, S. G. Kamita, N. Palekar, S. Volrath, E. Fowler, B. D. Hammock & S. Maeda. 1991. Development of a recombinant baculovirus expressing an insect-selective neurotoxin: potential for pest control. *Bio/Technology* 9: 848-852.
- McPherson, M. J., P. Quirke & G. R. Taylor. 1993. PCR: A Practical Approach. IRL Press at Oxford University Press, Oxford, England. 253 p.
- McLeod, P. J., S. Y. Young & W. C. Yearian. 1982. Application of a baculovirus of *Pseudoplusia includens* to soybean: efficacy and seasonal persistence. *Environ. Entomol.* 11: 412-416.
- Mehta, P., J. A. Wyman, M. K. Nakhla & D. P. Maxwell. 1994. Polymerase chain reaction detection of viruliferous *Bemisia tabaci* (Homoptera: Aleyrodidae) with two tomato-infecting geminiviruses. *J. Econ. Entomol.* 87: 1285-1290.
- Merryweather, A. T., U. Weyer, M. P. G. Harris, M. Hirst., T. Booth & R. D. Possee. 1990. Construction of genetically engineered baculovirus insecticides containing the *Bacillus thuringiensis* subsp. *kurstaki* HD-73 delta endotoxin. *J. Gen. Virol* 71: 1535-1544.

- Miller, L. K. 1981. A virus vector for genetic engineering in invertebrates. *In: Genetic Engineering in the Plant Sciences*. N. J. Panopoulos, Ed. Praeger Scientific, New York. pp. 203-224.
- Miller, L. K. 1988. Baculovirus as gene expression vectors. *Ann. Rev. Microbiol.* 42: 177-199.
- Miller, L. K. & K. P. Dawes. 1978. Restriction endonuclease analysis for the identification of baculovirus pesticides. *Appl. Environ. Microbiol.* 35: 411-421.
- Miller, L. K., J. E. Jewell & D. Browne. 1981. Baculovirus induction of a DNA polymerase. *J. Virol.* 40: 305-308.
- Miller, L. K., A. J. Ling, & L. A. J. Bulla. 1983. Bacterial, viral, and fungal insecticides. *Science* 219: 715-721.
- Minion, F. C., L. B. Coons & J. R. Broome. 1979. Characterization of the polyhedral envelope of the nuclear polyhedrosis virus of *Heliothis virescens*. *J. Invertebr. Pathol.* 34: 303-307.
- Mohamed, M. A., H. C. Coppel & J. D. Podgwaite. 1982. Persistence in soil and on foliage of nucleopolyhedrosis virus of the European Pine Sawfly, *Neodiprion sertifer* (Hymenoptera: Diprionidae). *Environ. Entomol.* 11: 1116-1118.
- Möller, A. & J. K. Jansson. 1997. Quantification of genetically tagged cyanobacteria in Baltic sea sediment by competitive PCR. *Biotechniques* 22: 512-518.
- Moraes, R.R. & Maruniak, J.E. 1997. Detection and identification of multiple baculoviruses using the polymerase chain reaction (PCR) and restriction endonuclease analysis. *J. Virol. Methods* 63: 209-217.
- Moscardi, F. 1989. Use of viruses for pest control in Brazil: The case of the nuclear polyhedrosis virus of the soybean caterpillar, *Anticarsia gemmatilis*. *Mem. Inst. Osw. Cruz, Rio de Janeiro* 84: 51-56.
- Moscardi, F., G. E. Allen & G. L. Green. 1981. Control of the velvetbean caterpillar by nuclear polyhedrosis virus and insecticides and impact of treatment of the natural incidence of the entomopathogenic fungus *Nomuraea rileyi*. *J. Econ. Entomol.* 74: 480-485.
- Moscardi, F. & D. R. Sosa-Gomez. 1992. Use of viruses against soybean caterpillars in Brazil. *In: Pest Management in soybean*. L.G. Copping, M. B. Green & R. T. Reeds, Eds. Elsevier, England. pp 98-109.

- Moscardi, F. & D. R. Sosa-Gomez. 1993. A case study in biological control: soybean defoliating caterpillars in Brazil. *In*: International Crop Science. I. D. R. Buxton, R. Shibles, R. A. Forsberg, B. L. Blad, K. H. Asay, G. M. Paulsen, & R.F. Wilson, Eds. Crop Science Society of America, Inc. Madison, Wisconsin, USA. p. 115-119.
- Munford, R. A., I. Barker & K. R. Wood. 1996. An improved method for the detection of Tospoviruses using the polymerase chain reaction. *J. Virol. Methods* 57: 109-115.
- Murphy, F. A., C. M. Fauquet, D. H. L. Bishop, S. A. Ghabrial, A. W. Jarvis, G. P. Martelli, M. A. Mayo & M. D. Summers. 1995. *Virus taxonomy*. Springer-Verlag Wien, New York, NY. pp. 104-113.
- Nagamine, T., H. Sugimori, K. Nakamura, S. Saga & M. Kobayashi. 1991. Nucleotide sequence of the gene coding for p40, an occluded virion-specific polypeptide of *Bombyx mori* nuclear polyhedrosis virus. *J. Invertebr. Pathol.* 58: 290-293.
- Naser, W. L. & H. G. Miltenburger. 1982. A rapid method for the selective identification of *Autographa californica* nuclear polyhedrosis virus using a monoclonal antibody. *FEMS Microbiology Letter* 15: 261-264.
- Naser, W. L. & H. G. Miltenburger. 1983. Rapid baculovirus detection, identification, and serological classification by western blotting-ELISA using a monoclonal antibody. *J. Gen. Virol.* 64: 639-647.
- Nataraju, B., V. Sivaprasad, R. K. Datta, S. K. Gupta & M. Shamim. 1994. Colloidal textile dye-based dipstick immunoassay for the detection of nuclear polyhedrosis virus (BmNPV) of silkworm, *Bombyx mori* L. *J. Invertebr. Pathol.* 63: 135-139.
- Nissen, M. S. & P. D. Friesen. 1989. Molecular analysis of the transcriptional regulatory region of an early baculovirus gene. *J. Virol.* 63: 493-503.
- Ohba, M., M. D. Summers, P. Hoops & G. E. Smith. 1977. Immunoradiometric assay for baculovirus enveloped nucleocapsids and polyhedrin. *J. Invertebr. Pathol.* 30: 362-368.
- Ooi, B. G., C. Rankin & L. K. Miller. 1989. Downstream sequences augment transcription from the essential initiation site of a baculovirus polyhedrin gene. *J. Mol. Biol.* 210: 721-736.
- O'Reilly, D. R., A. M. Crawford & L. K. Miller. 1989. Viral proliferating cell nuclear antigen. *Nature* 337: 606.

- O'Reilly, D. R. & L. K. Miller. 1989. A baculovirus blocks insect molting by producing ecdysteroid UDP-glucosyl transferase. *Science* 245: 1110-1112.
- O'Reilly, D. R. & L. K. Miller. 1990. Regulation of expression of a baculovirus ecdysteroid UDP-glucosyl transferase gene. *J. Virol.* 64: 1321-1328.
- O'Reilly, D. R. & L. K. Miller. 1991. Improvement of a baculovirus pesticide by deletion of the EGT gene. *Biotechnology* 9: 1086-1089.
- Paschke, J. D. & M. D. Summers. 1975. Early events in the infection of the arthropod gut by pathogenic insect viruses. *In: Invertebrate Immunity*. K. Maramorosch & R. E. Shope, Eds. Academic Press, New York. p. 75.
- Pavan, O. H. O. & H. C. T. Ribeiro. 1989. Selection of a baculovirus strain with a bivalent insecticidal activity. *Mem. Inst. Oswaldo Cruz* 84: 63-65.
- Payne, C. C. 1982. Insect viruses as control agents. *Parasitology* 84: 35.
- Pearson, M., R. Bjornson, G. Pearson & G. Rohrmann. 1992. The *Autographa californica* baculovirus genome: Evidence for multiple replication origins. *Science*, 257: 1382-1384.
- Pearson, M. N. & G. Rohrmann. 1995. *Lymantria dispar* nuclear polyhedrosis virus homologous regions: Characterization of their ability to function as replication origins. *J. Virol.* 69: 213-221.
- Picard, C., C. Ponsonnet, E. Paget, X. Nesme & P. Simonet. 1992. Detection and enumeration of bacteria in soil by direct DNA extraction and polymerase chain reaction. *Appl. Environ. Microbiol.* 58: 2717-2722.
- Podgwaite, J. D. & H. M. Mazzone. 1981. Development of insect viruses as pesticides: the case of the gypsy moth (*Lymantria dispar*, L.) in North America. *Protect. Ecol.* 3: 219-223.
- Podgwaite, J. D., K. S. Shields, R. T. Zerillo & R. B. Bruen. 1979. Environmental persistence of the nucleopolyhedrosis virus of the gypsy moth, *Lymantria dispar*. *Environ. Entomol.* 8: 528-536.
- Quant, R. L., M. N. Pearson, G. Rohrmann & G. S. Beaudreau. 1984. Production of polyhedrin monoclonal antibodies for distinguishing two *Orgyia pseudotsugata* baculoviruses. *Appl. Environ. Microbiol.* 48: 732-736.
- Raeymaekers, L. 1993. Quantitative PCR: theoretical considerations with practical implications. *Anal. Biochem.* 214: 582-585.

- Ramelow, C., J. Stüss, D. Berndt, M. Roggendorf & E. Schreier. 1993. Detection of tick-borne encephalitis virus RNA in ticks (*Ixodes ricinus*) by the polymerase chain reaction. *J. Virol. Methods* 45: 115-119.
- Rankin, C., B. G. Ooi & L. K. Miller. 1988. Eight base pairs encompassing the transcriptional start point are the major determinant for baculovirus polyhedrin gene expression. *Gene* 70: 39-49.
- Ravaggi, A., M. R. Biasin, D. Infantolino & E. Cariani. 1997. Comparison of competitive and non-competitive reverse transcription-polymerase chain reaction (RT-PCR) for the quantification of hepatitis C virus (HCV) RNA. *J. Virol. Methods* 65: 123-129.
- Ribeiro, B. M. & N. Crook. 1993. Expression of full-length and truncated forms of crystal protein genes from *Bacillus thuringiensis* subsp. *Kurstaki* in a baculovirus and pathogenicity of the recombinant viruses. *J. Invertebr. Pathol.* 62: 121-130.
- Richardson, C. D. 1995. *Baculovirus Expression Protocols*. Humana Press Inc., Totowa, New Jersey. 418 pp.
- Richter, A. R. & J. R. Fuxa. 1984. Timing, formulation, and persistence of a nuclear polyhedrosis virus and a microsporidium for control of the velvetbean caterpillar (Lepidoptera:Noctuidae) in soybeans. *J. Econ. Entomol.* 77: 1299-1306.
- Riegel, C. I., C. Lanner-Herrera & J. M. Slavicek. 1994. Identification and characterization of the ecdysteroid UDP-glucosyltransferase gene of *Lymantria dispar* multinucleocapsid nuclear polyhedrosis virus. *J. Gen. Virol.* 75: 829-838.
- Rodems, S. M. & P. D. Friesen. 1993. The hr5 transcriptional enhancer stimulates early expression from the *Autographa californica* nuclear polyhedrosis virus genome but is not required for virus replication. *J. Virol.* 67: 5776-5785.
- Rodems, S. M. & P. D. Friesen. 1995. Transcriptional enhancer activity of hr5 requires dual palindrome half sites that mediate binding of dimeric form of the baculovirus transregulator IE1. *J. Virol.* 69: 5368-5375.
- Rohrmann, G. F. 1986. Polyhedrin structure. *J. Gen. Virol.* 67: 1499-1513.
- Rohrmann, G. F. 1992. Baculovirus structural proteins. *J. Gen. Virol.* 73: 749-761.

- Roussel, J., M. E. Blair Zajdel, P. D. Howdle & G. E. Blair. 1993. Rapid detection of enteric adenoviruses by means of the polymerase chain reaction. *Journal of Infection* 27: 271-275.
- Sambrook, J., E. F. Fritsch & T. Maniatis. 1989. *Molecular Cloning: A Laboratory Manual*, 2nd ed. New York, Cold Spring Harbor Laboratory.
- SAS Institute. 1989. *SAS/STAT user's guide*, version 6, 4th ed., vol. 1-2. SAS Institute, Cary, NC.
- Schneeberger, C., P. Speiser, F. Kury & R. Zeillinger. 1995. Quantitative detection of reverse transcriptase-PCR products by means of a novel and sensitive DNA stain. *PCR Methods Appl.* 4: 234-238.
- Scott, H. A., W. C. Yearian & S. Y. Young. 1976. Evaluation of the single radial diffusion technique for detection of nuclear polyhedrosis virus (NPV) infection in *Heliothis zea*. *J. Invertebr. Pathol.* 28: 229-232.
- Shuler, M. L., H. A. Wood, R. R. Granados & D. A. Hammer. 1995. Baculovirus expression systems and biopesticides. John Wiley & Sons, Inc., New York, NY. 259 pp.
- Slavicek, J. M., J. Podgewaite & C. Lanner-Herrera. 1992. Properties of two *Limantria dispar* nuclear polyhedrosis virus isolates from the microbial pesticide Gypchek. *J. Invertebr. Pathol.* 59: 142-148.
- Slavicek, J. M., N. Hayes-Plazolles & M. E. Kelly. 1995. Rapid formation of few polyhedra mutants of the *Lymantria dispar* multi-nucleocapsid nuclear polyhedrosis virus during serial passage in cell culture. *Biological Control* 5: 251-261.
- Smirnov, W. A. 1975. Parasites and predators as vectors of insect viruses. *In: Baculoviruses for insect pest control: safety considerations*. Summers, M. D., R. Engler, L. A. Falcon & P. Vail, Eds. American Society for Microbiology, Washington, DC. pp. 131-133.
- Smith, G. E. & M. D. Summers. 1978. Analysis of baculovirus genomes with restriction endonucleases. *Virology* 89: 517-527.
- Smith, G. E. & M. D. Summers. 1981. Application of a novel radioimmunoassay to identify baculovirus structural proteins that share interspecies antigenic determinants. *J. Virol.* 39: 125-137.

- Smith, G. E., M. D. Summers & M. J. Fraser. 1983. Production of human β -interferon in insect cells infected with a baculovirus expression vector. *Molecular and Cellular Biology* 3: 2156-2165.
- Summers, M. D. 1977. Characterization of shrimp baculovirus. Ecological research series EPA-600/3-77-130, Environmental Protection Agency, Washington, D.C.
- Summers, M. D., R. Engler, L. A. Falcon & P. Vail. 1975. Baculoviruses for insect pest control: safety considerations. American Society for Microbiology, Washington, DC.
- Summers, M. D. & G. E. Smith. 1976. Comparative studies of baculovirus granulins and polyhedrins. *Intervirology* 6: 168-180.
- Summers, M. D. & G. E. Smith. 1978. Baculovirus structural polypeptides. *Virology* 84: 390-402.
- Summers, M. D. & G. E. Smith. 1987. A manual of methods for baculovirus vectors and insect cell culture procedures. Texas Agric. Exp. Stn.
- Summers, M. D., L. E. Volkman & C. H. Hsieh. 1978. Immunoperoxidase detection of baculovirus antigens in insect cells. *J. Gen. Virol.* 40: 545-557.
- Stewart, L. M. D., M. Hirst, M. L. Ferber, A. T. Merryweather, P. J. Cayley & R. D. Possee. 1991. Construction of an improved baculovirus insecticide containing an insect-specific toxin gene. *Nature* 352: 85-88.
- Stoltz, D. B., C. Pavan & A. B. da Cunha. 1973. Nuclear polyhedrosis virus: a possible example of the novo intranuclear membrane morphogenesis. *J. Gen. Virol.* 19: 145-150.
- Straub, T. M., I.L. Pepper, M. Abbaszadegan & C. P. Gerba. 1994. A method to detect enteroviruses in sewage sludge-amended soil using the PCR. *Appl. Environ. Microbiol.* 60: 1014-1017.
- Tanada, Y. 1964. A granulosis virus of the codling moth, *Carpocapsa pomonella*, (Linnaeus) (Olethreutidae, Lepidoptera). *J. Insect Pathol.* 6: 378-381.
- Tanada, Y. & R. T. Hess. 1976. Development of a nuclear polyhedrosis virus in midgut cells and penetration of the virus into the hemocoel of the armyworm, *Pseudaletia unipuncta*. *J. Invertebr. Pathol.* 28: 67-76.
- Tanada, Y. & H. K. Kaya. 1993. DNA-viral infections: Baculoviridae. *In: Insect Pathology*. Academic Press, Inc., San Diego, Ca. 666 p.

- Taylor, G. R. 1993. Polymerase chain reaction: basic principles and automation. *In*: PCR: A Practical Approach. McPherson, M. J., P. Quirke & G. R. Taylor, Eds. IRL Press at Oxford University Press, Oxford, England. pp. 1-14.
- Tebbe, C. C. & W. Vahjen. 1993. Interference of humic acids and DNA extracted directly from soil in detection and transformation of recombinant DNA from bacteria and a yeast. *Appl. Environ. Microbiol.* 59: 2657-2665.
- Theilmann D. A. & S. Stewart. 1992. Tandemly repeated sequence at the 3' end of the IE-2 gene of the baculovirus *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus is an enhancer element. *Virology* 187: 97-106.
- Thompson, C. G. & D. W. Scott. 1979. Production and persistence of the nuclear polyhedrosis virus of the dougla-fir tussock moth, *Orgyia pseudotsugata* (Lepidoptera: Lymantriidae), in the forest ecosystem. *J. Invertebr. Pathol.* 33: 57-61.
- Thomson, D. & R. G. Dietzgen. 1995. Detection of DNA and RNA plant viruses by PCR and RT-PCR using a rapid virus release protocol without tissue homogenization. *J. Virol. Methods* 54: 85-95.
- Tomalski, M. D. & L. K. Miller. 1991. Insect paralysis by baculovirus-mediated expression of a mite neurotoxin gene. *Nature* 352: 82-85.
- Tomalski, M. D., J. Wu & L. K. Miller. 1988. The location, sequence, transcription, and regulation of a baculovirus DNA polymerase gene. *Virology* 167: 591-600.
- Traverner, M. P. & E. F. Connor. 1992. Optical enumeration technique for detection of baculoviruses in the environment. *Environ. Entomol.* 21: 307-313.
- Tsai, Y. -L. & B. H. Olson. 1992. Rapid method for separation of bacterial DNA from humic substances in sediments for polymerase chain reaction. *Appl. Environ. Microbiol.* 58: 2292-2295.
- van der Wilk, F., J. W. M. van Lent & J. M. Vlask. 1987. Immunogold detection of polyhedrin, p10 and virion antigens in *Autographa californica* nuclear polyhedrosis virus-infected *Spodoptera frugiperda* cells. *J. Gen. Virol.* 68: 2615-2623.
- van Strien, E. A., D. Zuidema, R. W. Goldbach & J. M. Vlask. 1992. Nucleotide sequence and transcriptional analysis of the polyhedrin gene of *Spodoptera exigua* nuclear polyhedrosis virus. *J. Gen. Virol.* 73: 2813-2821.

- Vasconcelos, S. D., T. Williams, R. Hails & J. S. Cory. 1996. Prey selection and baculovirus dissemination by carabid predators of Lepidoptera. *Ecol. Entomol.* 21: 98-107.
- Vlak, J. M., F. A. Klinkenberg, K. J. M. Zaal, M. Usmany, E. C. Klinge-Roode, J. B. F. Geervliet, J. Roosien & J. W. M. van Lent. 1988. Functional studies on the p10 gene of *Autographa californica* nuclear polyhedrosis virus using a recombinant expressing a p10-beta-galactosidase fusion gene. *J. Gen. Virol.* 69: 765-776.
- Volkman, L. E. 1983. Occluded and budded *Autographa californica* nuclear polyhedrosis virus: immunological relatedness of structural proteins. *J. Virol.* 46: 221-226.
- Volkman, L. E. & P. A. Goldsmith. 1984. Budded *Autographa californica* NPV 64k protein: further biochemical analysis and effects of post-immunoprecipitation sample preparation conditions. *Virology* 139: 295-302.
- Volkman, L. E. & P. A. Goldsmith. 1985. Mechanism of neutralization of budded *Autographa californica* nuclear polyhedrosis virus by a monoclonal antibody: inhibition of entry by adsorptive endocytosis. *Virology* 143: 185-195.
- Volkman, L. E. & B. A. Keddle. 1990. Nuclear polyhedrosis virus pathogenesis. *Virology* 1: 249-256.
- Volkman, L. E. & M. D. Summers. 1977. *Autographa californica* nuclear polyhedrosis virus: comparative infectivity of the occluded, alkali-liberated, and nonoccluded forms. *J. Invertebr. Pathol.* 30: 102-103.
- Volkman, L. E., M. D. Summers & C. H. Hsieh. 1976. Occluded and nonoccluded nuclear polyhedrosis virus grown in *Trichoplusia ni*: comparative neutralization, comparative infectivity, and in vitro growth studies. *J. Virol.* 19: 820-832.
- Volossiuk, T., E.J. Robb & R. N. Nazar. 1995. Direct DNA extraction for PCR-mediated assays of soil organisms. *Appl. Environ. Microbiol.* 61: 3972-3976.
- Ward, V. K., S. B. Fleming & J. Kalmakoff. 1987. Comparison of a DNA-DNA dot-blot hybridization assay with light microscope and radioimmunoassay for the detection of a nuclear polyhedrosis virus. *J. Virol. Methods* 15: 65-73.
- Webb, A. C., M. K. Bradley, S. A. Phelan, J.Q. Wu & L. Gehrke. 1991. Use of the polymerase chain reaction for screening and evaluation of recombinant baculovirus clones. *Biotechniques* 4: 512-519.

- Webb, S. E. & A. M. Shelton. 1990. Effect of age structure on the outcome of viral epizootics in field populations of imported cabbageworm (Lepidoptera:Pieridae). *Env. Entomol.* 19: 111-116.
- Whitford, M. & P. Faulkner. 1992. Nucleotide sequence and transcriptional analysis of a gene encoding gp41, a structural glycoprotein of the baculovirus *Autographa californica* nuclear polyhedrosis virus. *J. Virol.* 66: 4763-4768.
- Whitt, M. A. & J. S. Manning. 1988. A phosphorylated 34-kDa protein and a subpopulation of polyhedrin are thiol linked to the carbohydrate layer surrounding a baculovirus occlusion body. *Virology* 163: 33-42.
- Wiedmann, M., R. Brandon, P. Wagner, E. J. Dubovi & C. A. Batt. 1993. Detection of bovine herpesvirus-1 in bovine semen by a nested PCR assay. *J. Virol. Methods* 44: 129-140.
- Wieland, U., H. Suhr, B. Salzberger, H. J. Eggers, R. W. Braun & J. E. Kühn. 1996. Quantification of HIV-1 proviral DNA and analysis of genomic diversity by polymerase chain reaction and temperature gradient gel electrophoresis. *J. Virol. Methods* 57: 127-139.
- Williams, G. V., D. Z. Rohel, J. Kuzio & P. Faulkner. 1989. A cytopathological investigation of *Autographa californica* nuclear polyhedrosis virus p10 gene function using insertion/deletion mutants. *J. Gen. Virol.* 70: 187-202.
- Wilson, W. C. & C. C. L. Chase. 1993. Nested and multiplex polymerase chain reactions for the identification of bluetongue virus infection in the biting midge, *Culicoides variipennis*. *J. Virol. Methods* 45: 39-47.
- Wood, H.A. 1980. Protease degradation of *Autographa californica* nuclear polyhedrosis virus proteins. *Virology* 103: 392-399.
- Wood, H. A., P. R. Hughes & A. Shelton. 1994. Field studies of the co-occlusion strategy with a genetically altered isolate of the *Autographa californica* nuclear polyhedrosis virus. *Environ. Entomol.* 23: 212-219.
- Xie, W.D., B. Arif, P. Dobos & P. J. Krell. 1995. Identification and analysis of a putative origin of DNA replication in the *Choristoneura fumiferana* multinucleocapsid nuclear polyhedrosis virus genome. *Virology* 209: 409-419.
- Young, S. Y. & T. J. Kring. 1991. Selection of healthy and nuclear polyhedrosis virus infected *Anticarsia gemmatilis* [Lep.: Noctuidae] as prey by nymphal *Nabis roseipennis* [Hemiptera: Nabidae] in laboratory and on soybean. *Entomophaga* 2: 265-273.

- Young, S. Y., J. R. Lack & W. C. Yearian. 1987. Transmission of a nuclear polyhedrosis virus in *Anticarsia gemmatilis* (Lepidoptera:Noctuidae) larval populations on caged soybeans. *Environ. Entomol.* 16: 691-698.
- Young, S. Y. & R. W. McNew. 1994. Persistence and efficacy of four nuclear polyhedrosis viruses for corn earworm (Lepidoptera: Noctuidae) on heading grain sorghum. *J. Entomol. Sci.* 29: 370-380.
- Young, S. Y. & W. C. Yearian. 1987. *Nabis roseipennis* adults (Hemiptera: Nabidae) as disseminators of nuclear polyhedrosis virus to *Anticarsia gemmatilis* (Lepidoptera:Noctuidae) larvae. *Env. Entomol.* 16: 1330-1333.
- Young, S. Y. & W. C. Yearian. 1989. Nuclear polyhedrosis virus infected and healthy *Anticarsia gemmatilis* larvae as prey for *Nabis roseipennis* adults in the laboratory. *J. Invertebr. Pathol.* 54: 139-143.
- Young, S. Y. & W. C. Yearian. 1990. Contamination of arthropod predators with *Heliothis* nuclear polyhedrosis virus after Elcar applications to soybean for control of *Heliothis* spp. (Lepidoptera: Noctuidae). *J. Entomol. Sci.* 3: 486-492.
- Young, S. Y. & W. C. Yearian. 1992. Movement of nuclear polyhedrosis virus into velvetbean caterpillar (Lepidoptera:Noctuidae) larval populations on soybean by *Nabis roseipennis* (Heteroptera:Nabidae) nymphs. *J. Entomol. Sci.* 27: 126-134.
- Zanotto, P. M. A., B. D. Kessing, & J. E. Maruniak. 1993. Phylogenetic interrelationships among baculoviruses: evolutionary rates and host associations. *J. Invertebr. Pathol.* 63: 147-164.
- Zanotto, P. M. A., M. J. A. Sampaio, D. W. Johnson, T. L. Rocha & J. E. Maruniak. 1992. The *Anticarsia gemmatilis* nuclear polyhedrosis virus polyhedrin gene region: Sequence analysis, gene product and structural comparisons. *J. Gen. Virol.* 73: 1049-1056.
- Zimmermann, K. & J. W. Mannhalter. 1996. Technical aspects of quantitative competitive PCR. *Biotechniques* 21: 268-279.
- Zuckerman, M. A., P. Levantis & J. S. Oxford. 1993. Direct sequence determination of the influenza B HA-1 gene after PCR amplification of clinical specimens from an infected volunteer. *J. Virol. Methods* 44: 35-44.
- Zuidema, D., E. C. Klinge-Roode, J. W. M. van Lent & J. M. Vlak. 1989. Construction and analysis of an *Autographa californica* nuclear polyhedrosis virus mutant lacking the polyhedral envelope. *Virology* 173: 98-108.

BIOGRAPHICAL SKETCH

Rejane Rocha de Moraes was born on 2 December 1963 in Pelotas, Rio Grande do Sul State, Brazil. She started Agronomy School in 1982 at the Federal University of Pelotas and completed her Bachelor of Science degree in 1986. In the same year, Rejane started her Master of Science program at the same University at the Entomology and Plant Pathology Department. She completed her M.S. in 1989. From December 1990 to May 1993, she worked at GERATEC Biotechnology Company as a production manager for a baculovirus-based biological insecticide.

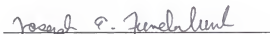
In August 1993, she started her Ph.D. program in the Department of Entomology and Nematology at the University of Florida, under the supervision of Dr. James E. Maruniak. Her Ph.D. project emphasizes the environmental detection of baculoviruses using the polymerase chain reaction technique.

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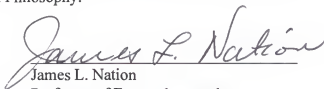
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August, 1997


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